1997 Annual Report of the Division of Intramural Research

National Institute of Child Health and Human Development

COVER

The image shows astrocytes from rat cerebral cortex in culture, stained with antibodies against glial fibrillary acidic protein (GFAP; green) and nestin (red). Some cells only express GFAP, whereas others express both nestin and GFAP (co-expression shows up as orange-yellow fluorescence). A significant number of astrocytes only express nestin, indicative of dedifferentiation. The image, made by Vittorio Gallo of the LCMN, was obtained by a double exposure of the same microscopic field on a Zeiss Axiophot fluorescence microscope (40×oil immersion lens).

Editor: Birgit An der Lan, Ph.D.
Photography: Bill Branson, Ernie Branson

For further information contact:

Arthur S. Levine, M.D.
Scientific Director
National Institute of Child Health and Human Development
National Institutes of Health
Building 31, Room 2A50
Bethesda, MD 20892-2425
Voice: 301 496 2133
Fax: 301 402 0105
E-mail: LevineA@hd03.nichd.nih.gov

NATIONAL INSTITUTE OF CHILD HEALTH

AND

HUMAN DEVELOPMENT

DIVISION OF INTRAMURAL RESEARCH

ANNUAL REPORT

1997

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

BOARD OF SCIENTIFIC COUNSELORS

Martha Constantine-Paton, Ph.D.	Professor of Biology, Yale University (Chair)
William W. Chin, M.D.	Chief, Division of Genetics, Department of Medicine, Brigham and Women's Hospital
Rosa M. Crum, M.D., M.H.S.	Assistant Professor of Epidemiology, Johns Hopkins Medical Institutions
Edward M.F. De Robertis, M.D., Ph.D.	Professor of Biological Chemistry and Norman Sprague Professor of Molecular Oncology, Department of Biological Chemistry, and Investigator, Howard Hughes Medical Institute, UCLA School of Medicine
Ronald W. Holz, Ph.D., M.D.	Professor of Pharmacology, University of Michigan Medical School
Gerald R. Fink, Ph.D.	Director, Whitehead Institute for Biomedical Research (Cambridge), and Professor of Molecular Genetics, MIT
Sally P. Mendoza, Ph.D.	Professor of Psychology, University of California (Davis), and Research Scientist, California Regional Primate Research Center
Robert G. Roeder, Ph.D.	Head, Laboratory of Biochemistry and Molecular Biology and Arnold O. and Mabel S. Beckman Professor, The Rockefeller University
Jess G. Thoene, M.D.	Professor of Pediatrics and Biological Chemistry and Director, Section of Biochemical Genetics and Metabolism, University of Michigan School of Medicine

CONTENTS

SCIENTIFIC DIRECTOR'S PREFACE

The Intramural Research Program is broadly concerned with the biological and neurobiological, medical and behavioral aspects of normal and abnormal human development. In addition to five major clinical research and training programs in the areas of genetics, endocrinology, and maternal-fetal medicine, a diversity of developmental models are under study in eighteen fundamental research Laboratories and Branches, drawing upon observations in bacteria, *Drosophila*, plants (*Arabidopsis*), yeasts, viruses, mollusks, zebrafish, frogs, rodents (including transgenic and "knock-out" mice), and subhuman primates. Disciplines employed in these studies include molecular biology, biochemistry, virology, immunology, pharmacology, genetics, cell and neuronal biology, biophysics, mathematical and theoretical biology, electrophysiology, reproductive physiology, structural biology, and developmental psychology. During the past year, we have emphasized a number of research projects that we believe have a particular promise and timeliness. In these areas, new resources have been added so that the investigators will be optimally poised to exploit their recent findings. These projects address aspects of the cellular and molecular biology of development (growth and differentiation) wherein we anticipate significant results with broad implications for biomedical research. Indeed, it is our notion that the central, and arguably the most exciting, questions in contemporary biology focus on the cellular and molecular mechanisms and interactions that guide a single fertilized egg cell through its development into a multicellular, highly organized and specialized adult organism.

The past year was an extremely productive one for this Intramural Research Program, and the year's scientific advances in each Laboratory and Branch are fully detailed on the following pages. Two new Laboratories were established in 1997—the Laboratory of Integrative and Medical Biophysics (LIMB) and the Laboratory of Structural and Physical Biology (LSPB). The investigators in these Laboratories, and their research resources, were transferred from the NIH's Division of Computer Resources and Training and the National Center for Research Resources after NIH leadership concluded that the research goals of the LIMB and LSPB scientists were more consistent with those of an intramural research program than a service organization. Dr. V. Adrian Parsegian is the Chief and two tenure-track scientists, Drs. S. Bezrukov and S. Leikin, complete the LSPB. The interests of this Laboratory are focused on the intracellular forces responsible for molecular conformation and assembly (including hydration forces responsible for channel dynamics), DNA-protein interactions, and the alteration of molecular conformation by electrical stimuli. Dr. Ralph Nossal is Chief of the LIMB, and the interests of this Laboratory are primarily in light scattering at a basic level, as well as the theoretical and applied basis of imaging of cells, tissues and organs (including MRI and other non-invasive techniques that rely on lasers). The LIMB includes two additional tenured scientists, Drs. R. Bonner and P. Basser. An important recent achievement in this Laboratory is the development of Laser Capture Microdissection, which permits the isolation of individual cells or groups of cells from complex tissues, to the end that the individual cell can be assayed for mRNA and protein expression, etc.

Late in the year, Dr. Richard Klausner asked to be relieved of his responsibilities as Chief, Cell Biology and Metabolism Branch (CBMB), given the transcendent challenges of his newer position as Director, NCI. Happily, Dr. Klausner's own immediate laboratory group will continue to function within the CBMB. Dr. Juan Bonifacino was appointed as the new Chief of this Branch. Bonifacino is an internationally recognized cell biologist, with a particular interest in the molecular signals that determine the assembly, routing, compartmentalization, and internalization of cellular proteins and multi-protein complexes. The appointment of Dr. Bonifacino to this important position reflects the institutional value that we have placed on cell biology, and the recognition that this very demanding, but rewarding, area of contemporary biomedical research profits greatly from the long-term support characteristics of the NIH's Intramural Program.

Also in 1997, we completed the construction of our new zebrafish facilities, and were fortunate to recruit two tenure-track scientists who are focused on this model for the study of vertebrate development. Drs. Brant Weinstein and Ajay Chitnis joined us after completing their post-doctoral fellowships at the Harvard Medical School; Dr. Weinstein's major interest is in the development of the cardiovascular system as well as hematopoietic differentiation, and Dr. Chitnis' major interest is in the development of the nervous system. We now have approximately 10,000 fish tanks, with an elegant facility proximate to the Laboratory of Molecular Genetics (LMG), and a satellite facility elsewhere on the campus. The zebrafish has the mutability of *Drosophila*, but demonstrates the classical stages of vertebrate embryogenesis; genetic screens should allow the

identification of hundreds, if not thousands, of hitherto unappreciated vertebrate gene functions. Moreover, a particular advantage of zebrafish is the optical clarity of the embryo.

During the past year, we completed the renovation of an additional corridor of space in Building 10, which has allowed expansion of the Laboratory of Cellular and Molecular Biophysics (Joshua Zimmerberg, M.D., Ph.D., Chief). In particular, this corridor provides new space in which to house this Laboratory's cooperative research program with NASA. This maneuver has also allowed the Institute to centralize all of its space in Building 10 on the 8th, 9th, and 10th Floors; heretofore, the labs in this Building have been quite dispersed. Locating the laboratory space on these floors also allows much closer proximity to our clinical research wards. The new space has also allowed the addition of a new independent research group led by Forbes D. Porter, M.D., Ph.D. in the Heritable Disorders Branch. During 1997, we also began construction of another building for the Laboratory of Molecular Embryology (LME)/CBMB complex, which will allow still further expansion of the research of both Laboratories, providing a significant core for Institute research in these rapidly growing and critical fields. The Laboratory of Eukaryotic Gene Regulation moved to vacated LME space, and in turn, vacated space in Building 6B, which permitted the addition of the two new zebrafish research groups in the LMG. The NICHD Intramural Research Program now comprises more than 150,000 sq. ft. of laboratory, office, and animal space.

In November, ground was broken for the NIH's Clinical Research Center, a 250-bed state-of-the-art hospital, which reflects rapidly evolving concepts of inpatient as well as outpatient care. The Building should be completed by 2002, at which time all of the Institute's patients, except those of the Perinatology Research Branch (PRB), will be housed in the new facility.

The PRB conducts clinical and laboratory research on factors responsible for perinatal morbidity and mortality, with a particular focus on those factors in high-risk urban populations. The Branch is emphasizing a multidisciplinary approach and utilizing the expertise from a number of clinical specialties (including obstetrics, perinatal pathology, neonatology, and diagnostic imaging), as well as from basic sciences and epidemiology/demography, to improve our etiologic understanding, diagnosis, treatment, and prevention of low birth weight and perinatal morbidity and mortality. The three elements of this intramural research Branch are located off-campus so that the Branch investigators have immediate access to a large population at risk for perinatal disorders. The primary Branch research site is therefore located in a regional academic medical center, and the Institute is currently negotiating a new infrastructure support contract designed to bring the Branch closer to the main NIH campus.

The Institute's vaccine development program continues to be exceptionally successful. Drs. John Robbins and Rachel Schneerson, who lead this program, were honored with the 1996 Albert and Mary Lasker Foundation Award for Clinical Research for their development of the *Haemophilus influenzae* conjugate vaccine. This vaccine, now used world-wide, has eliminated the most common cause of acquired mental retardation (a consequence of the meningitis commonly caused by this organism), saving at least one-half billion dollars yearly in U.S. health care costs. Indeed, this is one of the most important achievements, with respect to the public health, of any intramural scientific group at the NIH in modern times.

Notable also during 1997 were the appointments of two NICHD tenure-track investigators, Drs. Yoshihiro Nakatani and Leonid Chernomordik, to tenured positions. Dr. Nakatani's group is located within the Laboratory of Molecular Growth Regulation. His focus is on the molecular mechanisms of gene expression in the context of chromatin. Dr. Chernomordik's group is located within the Laboratory of Cellular and Molecular Biophysics, and his interests are in the mechanisms that underlie membrane fusion. Two scientists were appointed to the tenure track following international searches in 1997. Dr. Mary Lilly was recruited from the Carnegie Institution and will join the Cell Biology and Metabolism Branch. Her interest is in mechanisms that link developmental signals with cell cycle control, with a focus on the endocycle in *Drosophil*a. Dr. Tamas Balla's laboratory is within the Endocrinology and Reproduction Research Branch and he is primarily interested in signal transduction in endocrine cells, with a particular interest in the roles of calcium, phosphoinositols, and kinases in various signaling cascades.

A major issue confronting the NIH and the Nation as a whole is the recent and dramatic decline in patient-oriented research, i.e., research that requires interaction between a physician-investigator and a patient (in contrast to disease-oriented research in which, for example, a Ph.D. investigator might study oncogenes in the laboratory but not in patients *per se*). The number of physicians participating in patient-oriented research has fallen over the past decade for a number of reasons, while disease-oriented research, as informed by cell and

molecular biology, has been on the ascendancy. The managed care industry has had an impact on the numbers and types of patients enrolling as clinical research subjects; other factors underlying the decline in patient-oriented research include the increasing emphasis of medical schools on preparing their students for careers in primary health care delivery as opposed to research; attrition in the number of "role models" for young physicians; and the often great cost and multiple challenges inherent in patient-oriented research. Nonetheless, the opportunities in patient-oriented research have never been greater since disease-oriented research, primarily at the cellular and molecular levels, is providing extraordinary insight into the mechanisms underlying human disorders and diseases, which will surely lead to dramatic new therapeutic approaches, approaches that can only be realized with the participation of physician-investigators. Guided by this notion, and as discussed above, the NIH is embarking on the construction of an entirely new research hospital on the NIH campus to replace the aging Clinical Center. Moreover, the NIH is developing a diversity of new programs designed to attract medical students and young physicians, early in their careers, to the rich possibilities of a career in patient-oriented research. One such program, analogous to the Howard Hughes Medical Research Institute's program, which supports medical students in full-time laboratory research on this campus for one to two elective years, will offer support for medical students to spend a similar period in full-time patient-oriented research. Despite the complexity and unexpectedly rapid unfolding of the issues confronting patient-oriented research, the hope is that these several new efforts, as well as a growing awareness nationally of the serious implications of the decline, will ultimately reverse this unfortunate trend. Along these lines, we were especially pleased early in the year with visits to the NICHD's clinical research wards by Senator Kennedy and Dr. Shalala (Secretary, DHHS), accompanied by Dr. Varmus (Director, NIH). All three were deeply impressed by our presentation of NICHD's clinical research program.

During 1997, we also launched a new collaborative intramural-extramural clinical research activity, using the Smith-Lemli-Opitz Syndrome as a paradigm (SLO is a developmental disorder thought to be due to abnormal cholesterol metabolism and eventuating in mental retardation, a variety of congenital anomalies, and poor growth). SLO patient populations will be transferred from medical centers elsewhere in the country to the NICHD, and the appropriate investigators in these other centers will have sabbatical visits with us during which collaborative protocols will be implemented. This will maximize the use of our clinical resources, and develop opportunities for extramural investigators who might otherwise be limited by clinical resource limitations in their home institutions. This form of collaboration also ensures that we can build a critical patient population quickly, even in the face of a rare disorder, thus promoting more rapid research progress.

HIGHLIGHTS OF THE YEAR'S RESEARCH

Cell Biology and Metabolism Branch. Major research advances achieved in this Branch (CBMB) during the past year include further characterization of the molecular mechanisms of iron metabolism, particularly the role of the iron-sensing proteins known as iron-response element binding proteins (IRP1 and IRP2). IRP1 increases intracellular available iron by binding to ferritin and transferrin receptor mRNAs when iron levels are depleted, resulting in inhibition of ferritin mRNA translation and prolongation of the half-life of transferrin receptor mRNA. IRP1 displays cytosolic aconitase activity when not bound to RNA. IRP2 is a mitochondrial aconitase, which is less responsive to iron levels than IRP1.

Genetic studies in yeast are contributing to our knowledge of the mechanisms that control the uptake of metals such as iron, copper, and zinc by the cell, and have led to a model linking eukaryotic cellular iron uptake to copper uptake. The model comprises a cell-surface ferric reductase, an iron transporter, a copper oxidase, an intracellular copper-dependent ferrous ion oxidase, a plasma membrane copper transporter (CTR1), and a copper transporter that delivers copper to the iron oxidase (CCC2); this latter copper transporter is highly homologous to human copper-transporting ATPases, including those defective in Menke's and Wilson's disease. A single iron-sensing transcriptional regulator (AFT1) appears to coordinate the regulation of this cluster of genes by interacting with 5′ consensus binding sites. Branch members identified the nuclear protein MAC1 as a transducer of copper signals to the ferric reductase and the plasma membrane copper transporter. This system may well be paradigmatic for gut absorption of iron in mammals, and therefore relevant to the heritable human iron storage disorder, hemochromatosis. In this context, evidence was obtained that cell-cycle progression may be regulated by iron.

A novel protein complex named AP-3 that is involved in protein trafficking has been identified. Mutations in one of the subunits of this complex in *Drosophila* have been shown to result in defective pigment granule biogenesis, suggesting a role for the AP-3 complex in protein sorting to specialized endosomal-lysosomal organelles. *Drosophila* AP-3 mutants could serve as a model system to study the bases of some human

pigmentation disorders. CBMB workers have also established the sequence code for binding of address signals in the cytosolic tails of endocytic receptors and lysosomal membrane proteins to AP-3 and other components of the sorting machinery.

Members of the CBMB continue to characterize the mechanisms by which protein and membrane traffic in the cell are regulated. Evidence was obtained that proteins from the ER travel to the Golgi via an intermediate compartment and that Golgi-resident proteins can travel in a retrograde direction. Using various multi-component immune system proteins as models, work has also continued on the molecular determinants of protein retention, assembly, transport, endocytosis, and lysosomal targeting within the cell.

One CBMB group investigates the response of bacteria and plants to environmental signals. Treatment of *E. coli* with low doses of H_2O_2 results in the induction of an untranslated regulatory RNA denoted OxyS and a distinct group of proteins, which leads to resistance to subsequent higher doses of hydrogen peroxide. The expression of many of the H_2O_2-inducible genes is controlled by the redox-sensitive regulator OxyR. These workers have recently shown that the OxyS RNA acts as both a global regulator and an antimutator. They have also begun to elucidate the mechanism of redox-signaling by OxyR.

The plant *Arabidopsis* is utilized to determine how light signals are transduced into changes in plant growth and morphology. Mutant *Arabidopsis* seedlings, whose growth is not inhibited by blue light, are being characterized to gain insights into the signalling pathways that initiate normal photomorphogenesis.

Work continues on defining the tyrosine kinase based signaling pathways in lymphocytes. The ZAP-70 protein tyrosine kinase is critical to T cell antigen receptor-mediated activation. The properties of this enzyme were investigated extensively by reconstitution of wild-type and mutant forms into new ZAP-70-deficient T cells. Such studies revealed two pools of this enzyme: one that translocates from cytoplasm to the membrane upon cellular activation and a second, unexpected pool, which is nuclear. Study of the dynamic properties of this important molecule is under way. Characterization of tyrosine kinase substrates in these pathways reveals that Cbl, a complex adaptor protein that binds to many critical intracellular signaling proteins is also capable of blocking the activity of the Syk protein tyrosine kinase in mast cells. In this manner Cbl can also regulate mast cell function.

A project aimed at characterizing the function of the tumor suppressor gene VHL has brought the cloning of the VHL gene and the discovery that the VHL gene product associates with the RNA polymerase II-transcription elongation factor, elongin. Mutations in the VHL are associated with various inherited and spontaneous tumors, and it is thought that the VHL gene product may be a key element in controlling transcription elongation. To define the role of VHL, proteins that bind to the VHL/elongin complex were sought. One such protein is a so-called cullin, thought to be involved in cell cycle regulation. VHL's tumor restraining function appears to be related to its role in enabling cells to respond to nutritional signals and in suppressing the production of vascular endothelial growth factor.

One group has been performing genetic screens in *Drosophila* to identify factors that modulate transcription levels *in vivo*. These screens rely on dose-sensitive phenotypes that are caused by expression of transgenes under transcriptional control of eye-specific regulatory sequences. These studies have led to the identification of mutations in components of the basal transcription machinery as well as mutations in chromatin remodeling factors.

Developmental Endocrinology Branch. Studies in this Branch (DEB) have defined the role and mechanisms of action of insulin- like growth factors (IGFs) in fetal and postnatal development. IGF-I has been shown to function as an endogenous brain insulin, ensuring that highly metabolically active nerve cells obtain the nutrients they need during development. In the absence of local brain IGF production, mental retardation results due to stunting of neuronal processes and synaptogenesis. These workers have also shown that local IGF-I expression is essential for normal development of mature ovarian follicles and endometrial responses to steroids. In particular, IGF-I has been shown to be essential for mediating estradiol's mitogenic effects upon uterine tissues, with cells arrested in the G2 phase of the cell cycle in the absence of local uterine IGF expression.

Ongoing studies have emphasized the control of linear growth, both in humans and in animal models. Placebo-controlled clinical trials of recombinant human growth hormone (rGH) are in progress in children with idiopathic short stature and Turner's syndrome, and an attempt is being made to maximize the action of rGH by delaying epiphyseal closure.

Researchers in the DEB are also trying to understand the genetic and environmental factors that lead children, particularly those belonging to minority populations, to become overweight. These investigators have identified fundamental differences in metabolism and body composition, which help explain why African American girls gain weight more readily, and find it more difficult to lose weight, than Caucasian girls.

Another important project is to characterize the pathogenesis of spontaneous premature ovarian failure. During the past year investigators in the Branch determined that two-thirds of young women with spontaneous premature ovarian failure have a lower femoral neck bone mineral density (more than one SD below the mean) than a reference group and thus are at increased risk of hip fracture, despite receiving conventional hormone replacement therapy. Research projects to define the best strategies to build and maintain bone mass in these young women are now being planned. The group also investigated the utility of routine testing for other associated autoimmune endocrine disorders in these young women, and found that only screening for hypothyroidism and diabetes appears justified in patients with premature ovarian failure who desire fertility.

Other investigators in the DEB carry out mouse and human genetic studies to understand the pathogenesis of Non-Insulin-Dependent Diabetes Mellitus (NIDDM), a common metabolic disorder, which is estimated to affect about 10 million Americans. Using genetically engineered mice, they have shown that NIDDM is an oligogenic disease, i.e., it does not result from a major genetic abnormality at a single locus, but rather from the interaction of subtle genetic abnormalities in different genes involved in insulin action. In other studies, investigators in this group have established an important role for insulin receptors as growth-promoting receptors in embryonic development. Interestingly, this role appears to result from IGF-2 binding to the insulin receptor, rather than insulin itself, thus establishing a novel and important paradigm in biology, whereby the same receptor can exert different functions in response to different ligands.

Recently, Ca^{2+}-sensing receptor gene mutations were identified in patients with autosomal dominant and sporadic hypoparathyroidism. The insights gained from these studies have important implications for the genetic counseling and treatment of patients with this disease. Molecular studies of the mutations help define the mechanisms by which these G-protein coupled receptors transduce signals across the cell membrane. Other recent studies have explored the nutritional, endocrine, and paracrine factors regulating longitudinal bone growth. Basic knowledge in this area is applied to improve the diagnosis and treatment of growth disorders. Clinical trials are underway using growth hormone and insulin-like growth factor-1.

Ongoing work in the DEB has contributed to improved ability to determine the cause of Cushing's syndrome, through development of new criteria for interpretation of the metyrapone stimulation test, and development of a new test, jugular venous sampling, for the diagnosis of Cushing's disease.

Work in the DEB has contributed greatly to improving the accuracy of diagnosis and the effectiveness of treatment in Cushing's syndrome and other disorders characterized by abnormalities in CRH and cortisol secretion. It has been found that the hypothalamic CRH neuron is under the stimulatory influence of serotonergic, cholinergic, dopaminergic, and noradrenergic neurons, and under the inhibitory influence of the GABA/benzodiazepine and opioid peptide systems. While sequencing the regulatory region of the human CRH gene, DEB workers found functional estrogen response element consensus sequences, providing a possible explanation for the sexual dimorphism in stress responses and immunological regulation. Blunted responses to CRH administration indicative of low hypothalamic CRH secretion were found in patients suffering from the *post partum* blues/depression syndrome. The group's previous findings that CRH has peripheral inflammatory effects, opposite to those of its well-known central inhibitory action on the immune system, have now been extended to the demonstration that CRH-dependent inflammatory responses may play a role in ovarian, endometrial, and placental function; indeed, evidence has now been obtained that abnormal CRH secretion may be associated with ovarian pathology and premature labor. Studies on the molecular basis of inherited endocrine disorders continue to shed light on fundamental principles of hormone action. Thus, in patients with glucocorticoid and ACTH resistance syndromes, molecular defects of the glucocorticoid and ACTH receptors were elucidated, while in patients with the Carney complex multiple endocrine neoplasia syndrome two chromosomal loci harboring defective genes were mapped.

A topic of interest is adaptation of hypothalamic and pituitary ACTH responses to chronic stress. The findings point to the involvement of CRH receptors at the hypothalamic level, and interactive effects of CRH, vasopressin and glucocorticoids at the level of the pituitary corticotroph. Continuing studies on the physiological role of angiotensin II have shown that, in addition to its effects on cardiovascular homeostasis and cellular differentiation, the peptide regulates hypothalamic CRH expression and sympathoadrenal activity.

Endocrinology and Reproduction Research Branch. Members of this Branch (ERRB) have continued to explore the plasma membrane receptors for peptide and glycoprotein hormones, and the nature of the signal transduction systems that mediate their actions in endocrine cells in the hypothalamus, pituitary, gonads and adrenal glands. An analysis of the mechanisms of pulsatile GnRH release from normal and immortalized hypothalamic neurons revealed a novel and rapid inhibitory action of hCG on GnRH release that is G_i-mediated, which accounts for the suppression of pituitary gonadotropin secretion during pregnancy. The

autocrine actions of GnRH upon its neurosecretory cells of origin include concentration-dependent stimulatory and inhibitory effects on adenylate cyclase, as well as activation of inositol phosphate and calcium signaling pathways that influence pulsatile GnRH release. Regulatory elements for cell-specific expression of the GnRH receptor gene were identified in its promoter region, and cyclic AMP was found to stimulate gene expression. Several amino acids that are critical for normal G protein coupling, inositol phosphate/calcium signaling, and ligand-induced endocytosis were identified in the GnRH and angiotensin II receptors. In the angiotensin AT_1 receptor, two adjacent asparagine residues in the seventh transmembrane domain were found to be important for ligand binding and receptor activation, respectively. Studies on the mechanism of angiotensin action in adrenal glomerulosa cells have led to the isolation and molecular cloning of two novel wortmannin-sensitive PtdIns 4-kinases from the adrenal gland. These enzymes are essential for the maintenance of the agonist-sensitive polyphosphoinositide pools that provide the inositol phosphates mediating calcium signaling not only in the adrenal gland, but also in the many other ligand-activated cells in which the phosphoinositide/calcium pathway is operative.

Further studies on the LH receptor included an analysis of the functional role of the leucine-rich domains in its extracellular hormone binding region. In the human, an additional LH receptor gene was identified, cloned, and found to be regulated by cyclic AMP. Four copies of the human receptor gene were localized to chromosome 2p16-21, in contrast to a single copy in the rat. The expression of the rat prolactin receptor gene was found to be controlled by two tissue-specific promoters for the gonads and liver, and a common promoter that is operative in all prolactin target tissues and is the sole promoter in the mammary gland. The expression of the prolactin receptor within the gonads, but not in the liver and mammary gland, depends on the binding of steroidogenic factor 1 (SF-1) to its regulatory element in the gonad-specific promoter. In studies on the regulation of androgen synthesis, the activity of 17β-hydroxysteroid dehydrogenase was found to depend upon active glucose transport and the availability of ATP. The latter appears to be required for the formation of an activated phosphorylated form of the steroidogenic enzyme.

Another group has continued investigations on the role of voltage-gated and ATP-gated calcium influx on intracellular calcium homeostasis and hormone secretion in pituitary cells. In particular, the role of calcium influx in controlling of plasma membrane-associated potassium channels and endoplasmic reticulum-associated ryanodine-sensitive and inositol (1,4,5)-trisphosphate gated-channels, as well as prolactin and luteinizing hormone secretion, is being addressed.

In studies on regulation of neural functions by protein phosphorylation and oxidation, the *in vivo* states of phosphorylation and oxidation of two major protein kinase C (PKC) substrates in the brain, neurogranin and neuromodulin (GAP-43), were analyzed by immunochemistry and mass spectrometry. Phosphorylation and oxidation of these two proteins are thought to regulate the availability of calmodulin in neurons. Mutant mice that have a defective neurogranin gene are being used to investigate the physiological function of this protein.

Other investigators are exploring mechanisms of sulfonation, a process that is essential for normal growth and development as well as the maintenance of health. Current investigations involve the purification, characterization, cloning, and examination of the transcriptional regulation of novel steroid sulfotransferases, a growing family of biotransforming enzymes that play an important physiological role in the genomic and nongenomic action of steroid hormones. In the adrenal cortex, specific steroid sulfotransferases are differentially expressed by functionally distinct concentric zones. Using site-directed mutagenesis of the estrogen-specific enzyme, binding sites for the sulfonate donor and steroid acceptor substrates have been characterized. Importantly, these investigators have also purified, characterized, and cloned human and guinea pig PAPS synthase, the enzyme responsible for production of the universal sulfonate donor molecule, PAPS; and by recombinant DNA techniques, they have localized the catalytic domains of this crucial bifunctional protein. Furthermore, they have localized the gene for human PAPS synthase to chromosome 4, region q25-q26.

Heritable Disorders Branch. Members of the Heritable Disorders Branch (HDB) have undertaken genetic structure/function and biochemical studies of the collagen chains produced in various heritable disorders of connective tissue, e.g., *osteogenesis imperfecta* and Ehlers-Danlos syndrome. These studies have led to a regional model of collagen pathophysiology, and are the basis of attempts to develop selective antisense hammerhead ribozyme suppression of the mutant collagen allele, as a promising approach towards therapeutic intervention. Other therapeutic modalities being investigated include lower-limb bracing and growth hormone treatment. A non-lethal animal model of *osteogenesis imperfecta* is being generated by inserting suppressible collagen gene mutations into mice.

Work in this Branch has also emphasized heritable lysosomal storage diseases such as cystinosis, in which impaired transport of cystine out of lysosomes results in cellular destruction. The gene affected in cystinosis

patients has been mapped within two micro-satellite markers in a 580 kb region of the short arm of chromosome 17. In clinical studies, early diagnosis and treatment in infancy with cysteamine, which depletes cells of cystine, has been found to be extremely important; very early cysteamine treatment may, in fact, eliminate the need for renal transplantation in this disease. This team is also uncovering the molecular defects responsible for various types of Hermansky-Pudlak syndrome (HPS), an inherited pigment disorder, and is probing the role of the HPS protein in melanosome function.

Another important finding relates to the regulation of the human UDP-glucuronosyltransferase gene family. A defect in one such enzyme, bilirubin transferase, causes Crigler-Najjar (CN) Type I disease, a fatal hepatic disorder. Mapping of the UDP-GT genes has revealed a single-copy locus with a transcriptional arrangement comprising six nested promoters, allowing the one locus to produce six different enzymes. Newly identified exons expand the substrate specificity of enzymes encoded by the transferase locus. It was also found that the CN phenotype is caused by a range of mutations at this locus, all affecting bilirubin transferase activity. Analyses of these mutations and site-directed mutagenesis studies have enhanced our understanding of the structural determinants of these enzyme activities.

An important recent advance was the generation of a mouse model of human glycogen storage disease type 1a (GSD-1a), which is caused by a deficiency in glucose-6-phosphatase (G6Pase). The G6Pase-deficient mouse, which mimics the pathophysiology of human patients, will be used to understand the pathogenesis of GSD-1a, to delineate the mechanisms of G6Pase catalysis, and to develop somatic gene therapies for this disorder.

Work on uteroglobin (UG) gene-knockout in mice produced an animal model for a family of hereditary renal glomerular diseases. This disease is due to abnormal multimeric fibronectin deposit in the glomeruli. It may be caused by a mutation in the gene encoding either UG or its receptor. UG is an inhibitor of phospholipase A_2 which, like UG, has a cell surface receptor. Through this receptor, phospholipase A_2 transcriptionally activates cyclooxygenase-2 (COX-2), which facilitates the production of prostaglandins (PGs) from arachidonic acid (AA), and which is thought to play a key role in familial colorectal cancer. Work in this area has identified a nuclear factor (NF), NF-IL-6, as the critical element in phospholipase-A_2-induced COX-2 activation. Finally, the murine gene for palmitoyl protein thioesterase (PPT) has been cloned and characterized in order to generate an animal model for infantile ceroid lipofuscinosis (INCL) a uniformly fatal disease in children. INCL belongs to a larger group of a relatively common (1 in 12,500) hereditary neurodegenerative disease known as Batten disease, for which there is no effective treatment. An animal model for INCL may allow the development of novel and rational therapies for this serious disease.

The molecular basis of dysmorphic syndromes continues to be investigated. HDB workers are evaluating the role of *Lhx2* and *Lhx7*, two LIM homeobox genes, in development. Potential downstream targets of *Lhx2* have been identified, and a mouse model in which the *Lhx7* gene has been mutated is being produced. Progress has been made in identifying the molecular defect in the Smith-Lemli-Opitz syndrome by further characterizing cellular cholesterol metabolism in fibroblasts from these patients.

Laboratory of Cellular and Molecular Biophysics. By studying the biophysical mechanisms of protein-mediated membrane fusion, investigators in the LCMB hope to learn how the process of membrane fusion, which underlies viral entry, secretion, fertilization, and neuro-transmission, is catalyzed and regulated by biological organisms in health and in disease states. Using membrane fusion caused by the hemagglutinin of *influenza* (HA) as their paradigm, and software and hardware specially developed for the purpose of simultaneous measurement of electrical and fluorescent signals, they found that low temperature arrests fusion at a stage that precedes both lipid mixing and opening of a fusion pore but follows HA- and lipid-dependent local hemifusion of membrane monolayers restricted by a fence of activated HA surrounding the fusion site. This restricted hemifusion intermediate spontaneously transforms into complete fusion or stable hemifusion in HA-dependent manner. The HA fence will keep tension focused within the fusion site to allow the transformation of local hemifusion into an expanding fusion pore. If the number of activated HA molecules is not high enough to form a sufficiently large and tight complex (less fusion-competent HA, higher pH, or lower mobility of HA), or if the interactions between HA molecules in the complex are weaker (as in the case of GPI-HA 4), the growing hemifusion intermediate can break the HA fence and expand prior to the opening and expansion of a fusion pore. This model also explains LCMB's earlier data on the lipid sensitive stage of fusion prior to pore formation, a stage common to disparate biological fusion processes.

Sea urchin egg cortical granule (CG) fusion is a well characterized, highly pure, high-yield preparation for the study of docking and fusion events. The CGs retain their Ca^{2+}-sensitivity for fusion, either to one another, to isolated plasma membrane patches, and even to pure phospholipid vesicles, suggesting that the isolated granules carry with them all the molecular machinery necessary for docking, Ca^{2+}-sensing, and membrane-

membrane fusion. In a kinetic analysis of calcium-triggered exocytosis *in vitro*, LCMB investigators have extended a general, kinetic model that relates the rate and extent of triggered exocytosis to the number, distribution, and efficacy of activated fusion complexes by including an activation time for fusion complexes, have examined the calcium dependencies of the parameters described in this kinetic model, and have studied fusion kinetics in the presence of exogenous lipids known to inhibit and promote membrane fusion. A three-parameter model describes the fusion kinetics. The three parameters are the probability that a calcium-activated fusion complex becomes a committed fusion complex (q), the probability that a committed fusion complex fuses (p), and the average number of committed fusion complexes, ($<n>$). The relative invariance of $p/<n>$ with calcium is consistent with an intrinsic probability to fuse. The overall probability to fuse is dependent on the total number of committed fusion complexes. LCMB researchers have identified homologs of the mammalian fusion proteins VAMP2, SNAP-25, SNARE, and Syntaxin1A in CG membranes, further underscoring the utility of this paradigm

In other work, a novel technique for HIV infection of human lymphoid tissue ex *vivo* was developed, thus providing an invaluable tissue model in which to study HIV infection, for which there are no convenient animal models. It was established that in this system viral tropism is strongly correlated with $CD4^+$ T lymphocyte depletion, which enables one to study this hallmark of AIDS under controlled conditions ex *vivo*. In related work, the group is utilizing a rotating wall vessel originally developed by NASA for the Space Program as a bioreactor for pan-NIH collaborative histoculture projects.

Members of the LCMB also aim to develop systematic, rational approaches to separating subcellular particles, using separation theory and the physical properties of polymer media to predict optimally resolving conditions for each application, as well as to determine size, conformation, and surface charge of particles on the basis of their electrophoretic mobility. During the past year, isolation of such particles and their separation by size and shape were achieved in polymer solutions, with quantitative recovery. Advances were also documented in the theory of electrophoresis, particularly with regard to prediction of electrophoretic retardation, replacing purely mathematical modeling with experimentally verified models derived from polymer physics.

Another group in the LCMB employs mass spectrometry to analyze various molecules of biological significance. The group has recently acquired state-of-the-art mass spectrometric instrumentation, which is permitting it to determine very accurate values of molecular weights and partial sequences of proteins and peptides at very high sensitivity, using sources such as bands from gel electrophoresis. In addition, the group is investigating the fundamental aspects of hydrophobicity by determining the energetics of solvation of gas phase ions. Mass spectrometry techniques that are particularly suited for this purpose have been developed.

Laboratory of Cellular and Molecular Neurophysiology. This Laboratory (LCMN) is concerned with the mechanisms by which signal transduction occurs at a molecular level in the brain. At the vast majority of excitatory synapses in the mammalian CNS, glutamate activates AMPA receptors, which mediate fast synaptic responses, and relieves Mg block of NMDA receptors. Calcium flux through NMDA receptors then triggers cellular and genetic regulatory events that are thought to underlie memory, and the regulation of nerve cell growth and differentiation. Excessive activation of NMDA receptors leads to cell death during anoxia and stroke. LCMN workers have found that AMPA and kainate receptors show ion channel block by cytoplasmic polyamines and by polyamine toxins in insect venoms. An analysis of their mechanism of action shows that such toxins first enter and block the pore (open channel block), and then destabilize the open state via an allosteric mechanism forcing the channel to close and trapping toxin in the pore. Surprisingly, despite their large size the toxins are able to pass through the pore, raising the question of where the toxin binding sites are located. Block by external toxin is very efficient but requires open channels; although block by internal toxin occurs only at high concentrations and at a low rate, it develops in the absence of agonist suggesting an external gate.

A project that investigates the role of hippocampal inhibitory neurons in the regulation of neuronal excitability is based on the rationale that a single inhibitory interneuron may synapse with over 100 pyramidal neurons, and that modulation of hippocampal interneuron activity has the potential of being a powerful therapeutic tool in the management of seizures and other related pathophysiological disorders. The characterization of the physiological and pharmacological properties of these inhibitory neurons has continued, work over the past year focusing on their role in long-term potentiation and depression, on the kinetic properties of their potassium currents, on the nature of their potassium channels, and on mechanisms of excitatory postsynaptic transmission.

Work in this laboratory has also focused on the physiological role of neurotransmitter receptors, ligand-gated channels and voltage-dependent channels in glial cells. It was established that cultured oligodendroglial progenitor cells express non-NMDA glutamate receptors and noradrenergic β-receptors. Activation of both

receptor systems inhibits oligodendroglial progenitor proliferation by causing arrest in the G1 phase of the cell cycle, but has opposite effects on cell differentiation: glutamate receptor agonists prevent, whereas β-receptor agonists stimulate cell differentiation. In a more intact system (cerebellar slice cultures), glutamate receptor agonists decrease the number of oligodendrocyte progenitor cells and their proliferation, as well as the number of differentiated oligodendrocytes. A non-NMDA receptor antagonist has the opposite effects. mRNA transcripts of the oligodendrocyte gene 2',3'-cyclic nucleotide 3'-phosphodiesterase were significantly decreased by glutamate receptor agonists and increased by the antagonist. The gene encoding a glutamate receptor subunit of the kainate subtype and expressed in oligodendrocyte lineage cells was cloned. The 5'-flanking regions responsible for its tissue-specific expression were defined in cultured cells and in transgenic mice. A silencer present in intron 1 of this gene was characterized, and members of the nuclear orphan receptor family were found to bind to this silencer.

In order to investigate calcium-based excitability in glial cells in the brain, other members of the LCMN have developed methods to measure intracellular calcium signals in individual astrocytes. This year, they characterized astrocytic elementary calcium release sites (wave amplification sites), loci that support regenerative calcium wave propagation, and found that at these specialized calcium release sites there is an assembly of multiple cellular components, including a high density of Ins(1,4,5)P3 receptor channels and SERCA calcium pump sites on the endoplasmic reticulum membranes, high-density, bead-like accumulation of calreticulin in the endoplasmic reticulum lumen, and a close association with one or more mitochondria. They also characterized a calcium-activated potassium conductance on rat pineal cells, which is involved in modulation of excitation in these cells during circadian control of melatonin secretion.

Laboratory of Comparative Ethology. This Laboratory carries out basic research investigating biobehavioral development in humans and in nonhuman primates, with a major emphasis on characterizing interactions between genetic and environmental factors that affect the course of an individual's physiology and behavior throughout the life span. Research completed this past year identified significant differences in biobehavioral development among rhesus monkey infants reared by mothers who differed in chronic cerebrospinal (CSF) concentrations of 5-HIAA, the primary central metabolite of serotonin. Other studies described systematic associations between the relative incidence of different types of aggressive behavior and CSF levels of 5-HIAA and testosterone in free-ranging rhesus monkey adult males, and documented significant deficits in courtship and reproductive behavior in adolescent and young adult males with unusually low chronic CSF 5-HIAA concentrations. These workers also identified significant relationships between heart rate and adrenocortical responsiveness and specific aspects of maternal and group-directed social behavior in free-ranging multiparous adult rhesus monkey females. Lifespan longitudinal behavioral profiles generated for group-living captive rhesus monkeys revealed remarkable stability of both individual and gender-specific profiles from early adulthood to senescence despite significant changes in behavior as a function of increasing age. Research with capuchin monkeys characterized both short- and long-term predictive relationships between early infant behavioral and activity state profiles and subsequent biobehavioral developmental trajectories exhibited throughout late infancy and childhood. Other studies expanded the known range of complex tool-using behavior shown by capuchin monkeys and identified factors contributing to the dramatic individual differences in tool-use proficiency seen in this unusual primate species.

Another group has a long-term interest in the acoustical patterns, neuropharmacology, and neuroanatomy of primate vocalization, as well as the hormonal correlates of parenting behavior. This work has revealed that details in the acoustic structure of vocalizations from infant monkeys can serve as markers for individual, gender and rearing differences.

In studies on human infants, LCE investigators have previously demonstrated that three skills characteristic of cognitive functioning at five months of age (habituation, novelty preference, and cross-modal transfer) predict later childhood mental ability. It was found that variation in the information-processing ability of children may be explained by specific infant and maternal factors that are evident soon after birth. These studies are important in that they separate "nature from nurture," thus allowing assessments of the impact of a child's very early environment, positive or negative, and his or her genetic endowment on cognitive skills. In on-going cross-cultural analyses of mother-infant interactions, subtle differences were found that may correlate with different perceptions in various societies about parenting goals. Analyses of the correlates of language development in toddlers have revealed that nurturant maternal responsiveness is also a strong predictor of vocabulary size and pragmatic language use, as well as play skills, in infants and toddlers. The results of an important study on the effects of prenatal cocaine exposure indicate that exposed infants have attentional deficits and are more reactive than unexposed infants, and that exposure may result in slower motor, but not mental, development.

Another group within this Laboratory examines the effects of different types of care giving practices on children's social, emotional, and cognitive competence. One study involves a long-term follow-up of Swedish children, some of whom were enrolled at 16 months of age in either daycare centers or home daycare facilities. Previous analyses had demonstrated that the type of early child care arrangement did not predict individual differences in social skills, aggression, compliance, personality maturity, or cognitive achievement, whereas the quality of both early home care and early out-of-home care were reliable and strong predictors. By the time the children were seven or eight years of age, however, measures of personal maturity and verbal ability were significantly affected by the type of care received earlier. Home daycare was associated with the least mature personality and the poorest patterns of cognitive maturity, whereas center daycare was associated with superior patterns of performance. In a program of research concerned with the elicitation and evaluation of children's testimony about alleged incidents of sexual abuse, researchers also showed that open-ended questioning yielded longer and more detailed accounts than directive or leading questioning. These researchers then showed that alleged victims could be trained to produce more detailed responses simply by altering the ways in which investigators began their interviews. In a cautionary message, however, these researchers showed that popular measures of children's credibility were not reliable enough to be used forensically.

Laboratory of Developmental and Molecular Immunity. This Laboratory (LDMI) develops vaccines directed against important bacterial diseases, especially those of infants and children. To this end, the LDMI pioneered the development of a new generation of vaccines in which specific antigenic capsular polysaccharides are chemically conjugated to highly immunogenic but non-specific proteins. In the case of many bacteria, the only antigens capable of inducing protective immunity are capsular polysaccharides, but these polysaccharides are only weakly immunogenic, especially in the immature immune system. The new conjugate vaccines, which confer T-cell dependence and booster responses to polysaccharide antigens, entirely circumvent the problems of earlier vaccines, or allow the development of vaccines where none have existed previously. Because conjugate vaccines overcome the problems of a weak immune response in infants and children, they may also be useful in adults who are immuno-compromised, e.g., AIDS patients. Development by the LDMI of an *H. influenzae* type b conjugate vaccine, now employed throughout the world, has virtually eliminated *H. influenzae* type b meningitis (the most common cause of acquired mental retardation) wherever the vaccine has been used, including the United States. This achievement is indeed significant, certainly one of NIH's major contributions to public health.

The principles and methods underlying the *H. influenzae* vaccine have now yielded conjugate vaccines against *Salmonella typhi*, as well as vaccines directed against non-typhoidal enteric diseases (non-typhoidal *Salmonellae*, *Shigellae* and *Vibrio cholerae*), which are being field-tested. Previously, there were no practical nor highly effective vaccines for these common and serious intestinal pathogens. Three *Shigellae* conjugates (*dysenteriae* type 1, *flexneri* type 2a, and *sonnei*) have been synthesized and are undergoing clinical trials. The LDMI has also developed a conjugate vaccine for the prevention of Group 6 pneumococcus infection, a common cause of ear infections in infants and children and of systemic infections in sickle cell patients; the bacteria of this serogroup are now antibiotic-resistant. This vaccine is undergoing clinical trials in Iceland. Clinical trials of a conjugate *Staphylococcus aureus* vaccine are underway in patients with end-stage renal disease. Several novel approaches to conjugate vaccine development have been developed. One is to conjugate the detoxified lipopolysaccharide (the pyrogen) of pathogens with suitable proteins; this scheme has yielded two promising new vaccines, one against *Vibrio cholerae*, and one against *Salmonella typhimurium*. Another is to produce a non-toxic mutant shigella-like toxin and conjugate it to the capsular polysaccharide of *E. coli* 0157, the pathogen that causes the often fatal hemolytic uremic syndrome, especially in small children. A third is to produce a synthetic analog of the Vi antigen, the capsular polysaccharide of *Salmonella typhi*, by O-acetylation of pectin, a process that endows pectin with immunological properties similar to Vi.

Clinical trials, conducted in the midst of a severe whooping cough epidemic in Sweden, of the LDMI's monovalent pertussis toxoid vaccine has shown it to be entirely free of side-effects, highly effective in preventing pertussis, and capable of inducing "herd immunity" in a manner similar to that observed after vaccination against diphtheria. A mass vaccination program in Göteborg, in which half the children that were born in this decade (17,000) were injected with the monocomponent toxoid vaccine, has been completed with the result that there is no pertussis in that city. The current whole-cell vaccine (DPT) will soon be entirely replaced by the mono-component toxoid vaccine, which should confer both safety and specificity in the prevention of pertussis, and, if used in adults, should eliminate the remaining reservoir of this pathogen. Since the pertussis toxoid protein is a powerful immunogen, it can also be employed in the development of multivalent polysaccharide-

protein conjugate vaccines, leading to the possibility of infant immunization reagents that will protect against several infant diseases.

Laboratory of Developmental Neurobiology. Work in the LDN has continued on activity-dependent synapse elimination, with recent studies involving cholinergic neuron/muscle connections in a tissue culture chamber system, showing that synaptic connectivity can be regulated by external stimulation. Unstimulated synaptic connections to activated targets are selectively lost. It appears that the protease thrombin and the glia-derived protease inhibitor nexin I may be responsible for controlling circuitry during activity-dependent synapse elimination. The thrombin receptor and protein kinase C are essential for mediating activity-dependent synapse reductions. The group's expertise in this field is being applied to the study of synapse formation in the visual system.

Other workers in the LDN have sought to discover the molecular basis by which electrical activity influences the survival and differentiation of nerve cells in the central nervous system. This group was the first to demonstrate that vasoactive intestinal peptide (VIP) has a potent but indirect action in promoting the survival of developing neurons in culture, by acting as a secretagogue for neurotrophic molecules released from astrocytes. The secreted substances include protease nexin I, a recognized serine protease inhibitor with actions on neurite extension and neuronal survival, and cytokines, neuroimmune regulators with effects on many developmental functions. Another substance released by VIP, activity-dependent neurotrophic factor (ADNF), which demonstrates activity at femtomolar concentrations, has now been characterized and found to have a similar structure to a stress protein. A nine-amino-acid peptide derived from ADNF has been shown to protect neurons from clinically relevant neurotoxic substances, including N-methyl-D-aspartate (excitotoxicity), β-amyloid (Alzheimer's disease), and gp120, the envelope protein of the human immunodeficiency virus.

Work on the pineal gland mainly emphasizes how the production of melatonin is regulated by arylalkylamine N-acetyltransferase (AANAT) , an important enzyme in circadian biology. AANAT activity increases 10-100- fold at night in all vertebrates. Evidence was obtained that in some, but not all, vertebrates this requires an increase in transcription, which is driven by an endogenous clock. In some cases this clock is located in the pineal gland, and studies are under way to determine the link between this pineal clock and the gene encoding AANAT. A second important regulatory mechanism being investigated is very rapid AANAT proteolysis, which is normally inhibited by cyclic AMP in all vertebrates. Other areas of interest include the basis of tissue-specific regulation of AANAT and the development of drugs that inhibit enzyme activity and others that prevent its proteolysis.

Studies on the ACTH/endorphin/MSH family of peptides were also continued this year. These peptides are synthesized in the brain and pituitary from a common glycoprotein precursor (pro-opiomelanocortin, POMC). A conformation-dependent sorting signal in the N-terminal domain of POMC comprising a 13-residue, disulfide-stabilized loop with two pairs of acidic/hydrophobic residues exposed on the surface was identified as the signal for targeting POMC to the regulated pathway of neurosecretion; similar motifs were also found in other prohormones, suggesting that conformation-dependent sorting signals are an important element of prohormone secretion. The sorting receptor for this motif has been identified as membrane carboxypeptidase E. Processing of prohormones depends on proteolytic processing enzymes, the characterization of which has occupied this group of workers over the past few years. A novel class of prohormone processing aspartic proteases, with specificity for basic residues due to highly electronegative pockets in the active site, has now been found in mammals and yeast.

Other studies, using various clostridial neurotoxins, are helping to define synaptic vesicle membrane trafficking. These toxins are zinc endopetidases, which cleave proteins involved in synaptic vesicle docking and fusion. One such toxin, botulinum neurotoxin A, selectively blocks synaptic vesicle exocytosis, but not endocytosis, which were previously thought to be tightly coupled, establishing the toxin as a valuable tool to dissect these processes.

Other studies have focused on the molecular pathways that couple synaptic signaling to specific transcriptional regulatory pathways. A novel mechanism was discovered that may be of general importance for selectively regulating neural plasticity during development: the need for converging signals elicited by molecules that are released from presynaptic neurons to specifically regulate gene expression in the post-synaptic cell. The activation of tyrosine kinase receptors by neuregulins and of activity mediated by the opening of glutamate receptors were found to induce transcription of one type of NMDA receptor subunit while inhibiting the expression of another.

One team of researchers has been investigating how information coded in the pattern of action potential firing is transduced and integrated within the neuron to control expression of genes regulating the structure and

function of the nervous system. The work has shown that cell adhesion molecules, immediate early genes, and ion channels are regulated by specific patterns of action potentials. Functional activity regulates neurite outgrowth, axon fasciculation, cell adhesion, and interactions between neurons and glia through changes in expression of these molecules. Second messengers, kinases, and transcription factors regulating gene expression in response to action potentials are being identified, and the subcellular distribution and temporal features of these signaling pathways are being investigated. This research shows that kinetic differences in intracellular signaling pathways are critical in regulating certain genes in response to specific patterns of firing.

Another group in the Laboratory is investigating the roles of neurotrophic factors in synapse development and plasticity. These workers have demonstrated that the neurotrophin NT-3 plays an important role in the development of the neuromuscular synapses. They have also established that another neurotrophin, BDNF, facilitates the development of long-term potentiation in the hippocampus.

Laboratory of Eukaryotic Gene Regulation. A particular interest of this Laboratory (LEGR) is the nature of signals received by eukaryotic cells from their changing environment, and the mechanisms by which cells respond to these signals. The response of yeast to nutrient availability is a prime model for studying these reactions. As shown by LEGR investigators, the yeast response mechanism utilizes virtually all major molecular pathways, including transcriptional and translational regulation, as well as protein modification. In yeast, the absence of any one amino acid from the medium leads to increased production of the entire set of amino acid biosynthetic enzymes; this response is called general control, and the key regulatory molecule in the pathway is GCN4, a transcriptional activator of the genes encoding the biosynthetic enzymes. The fine structure of the GCN4 activation domain has been elucidated and its interactions with general transcription factors and coactivators are being probed using a combination of genetics and biochemistry. The *GCN4* gene is continuously transcribed, but GCN4 protein is only produced during amino acid starvation. Leading up to the choke point of general control, regulation of *GCN4* mRNA translation, is a hierarchy of *GCD* and *GCN* genes, whose positive and negative actions are required to achieve the described effects. Many of the GCD proteins are components of the general machinery for protein synthesis initiation, such that analysis of GCN4 translational control provides a window onto the molecular events involved in assembly of translation initiation complexes. *GCN2* encodes a kinase that phosphorylates the α-subunit of eIF2, a translation initiation factor that delivers initiator tRNA to the ribosome. The GCN2 protein has two components: a kinase domain, and a domain with substantial sequence homology to histidyl-tRNA synthetase; through its tRNA synthetase-like domain, GCN2 is able to sense the concentration of uncharged tRNAs in the cell, and use this information to regulate its kinase activity, thus connecting the nutritional state to *GCN4* regulation. It has been found that additional GCN proteins facilitate the activation of GCN2 by uncharged tRNA on the ribosome, and these ancillary factors, as well as GCN2 itself, are conserved between the fungal and animal kingdoms.

A related interest of the laboratory is the mechanism and regulation of protein biosynthesis in eukaryotic cells. Recent progress has provided insights into both substrate recognition and viral regulation of the mammalian protein kinase PKR that inhibits translation by phosphorylating the translation factor eIF2α. A novel mechanism of kinase inhibition has been identified in studies of an insect *Baculovirus*, which expresses a truncated kinase homolog that inhibits cellular eIF2α kinases. Also, a new yeast translation initiation factor, homologous to the bacterial translation factor IF2, has been characterized.

The mechanisms of retroelement propagation are another topic of interest. Particular emphasis is being placed on retrotransposons, genetic elements that bear many similarities to retroviruses, but exist in yeasts, which are genetically more tractable hosts than mammals. A newly developed retrotransposition assay has enabled the group to investigate the unique priming mechanism whereby cDNA synthesis is initiated in retrotransposons. Also, host genes have been identified that are required for transposition. One such gene product is associated with the nuclear pore and contributes to the nuclear import of Tf1 protein and cDNA.

Laboratory of Integrative and Medical Biophysics. The overall goal of this newly established Laboratory (LIMB) is to elucidate basic mechanisms of normal cell and tissue function, as well as to analyze alterations that occur in disease. The methods of the physical and engineering sciences are brought to bear on the development and application of quantitative methods for determining cell and tissue status. For example, during the past year, members of LIMB have devised a mathematical algorithm that enables one to invert diffuse time-gated light scattering data to infer the optical properties of targets buried deeply under the surface of an optically turbid medium such as biological tissue. This is the first theory that provides good measures of a target whose optical properties (scattering and absorptive) are close to those of the background. It has been applied successfully to time-resolved transillumination measurements of a multiply-scattering, tissue-like phantom. Not only was the size of the target determined to a good approximation, but the optical parameters were determined with high

accuracy. A similar reconstruction algorithm has been developed recently to quantify concentrations of fluorophores located at discrete sites below a tissue surface. That algorithm has also been successfully tested with data obtained from an artificial, tissue-like object, in this case designed to model fluorescently labeled salivary glands; this is part of an ongoing collaborative project to provide optical assessment of the autoimmune disease known as Sjogren's Syndrome.

Another group has pioneered the development of Laser Capture Microdissection (LCM) and its application to multiplex molecular analysis (DNA, mRNA, and protein) of specific homogeneous pools of cells associated with disease pathology. In collaboration with the NCI, staff members have developed the ability to microdissect pure homogeneous populations of cells from normal epithelia, early premalignant lesions, *in situ* carcinoma, and invasive cancer, all from the same surgical specimen, and compare both genetic alterations (DNA) and alterations in gene expression (mRNA) patterns. Through a Cooperative Research and Development Agreement (CRADA) with Arcturus Engineering, a commercial LCM computerized microscope has been developed for routine microdissection and, in collaboration with the NCI, a core LCM lab was established for the NIH community and outside researchers. The group is currently applying LCM combined with multiplex, quantitative molecular analysis using PCR, RT-PCR, microarray hybridization, and immunochemistry to the analysis and diagnosis of disease pathologies and to the determination of the temporal changes in patterns of gene expression during development and in normal organ function. A major new direction is to develop robust quantitative techniques for multiplex molecular analysis of microdissected cells suitable in clinical diagnosis, and to devise approaches for meaningful interpretation of highly complex interactions within gene expression patterns of hundreds of genes measured simultaneously.

A third group has continued to develop Diffusion Tensor Magnetic Resonance Imaging (DT-MRI) as a noninvasive imaging modality, and to apply it to characterization of microstructural features of normal, developing, and pathological tissues *in vivo*. This year, staff proposed and tested several new MRI parameters that behave like quantitative histological stains but require no exogenous contrast agents. One such parameter behaves like horseradish peroxidase, permitting one to visualize nerve fiber tracts *in vivo* at a resolution of about 1 mm^3. Collaborators in NINDS have applied DT-MRI methods to characterize fiber tract architecture in normal human brain, and assess its changes in development and disease (such as chronic stroke). This coming year, staff will perform DT-MRI at microscopic resolution, and develop new mathematical models of water transport in tissues so that one can infer mean cell volume, and other useful tissue characteristics, *in vivo*.

Members of LIMB reported the first measurement of the distensibility of the collagen network of cartilage. Using a novel osmotic stress titration technique, it was possible to show that the collagen network becomes flaccid in osteoarthritis.

Other projects undertaken in the Laboratory have been directed towards understanding biophysical aspects of the formation of biological vesicles such as those involved in intracellular transport. These vesicles are covered with protein coats, the best studied of which is a structure composed primarily of clathrin. A physical theory was developed to explain the size distributions of clathrin coats and to estimate intramolecular energies pertinent to coat formation. Biophysical studies were also conducted on the properties of supramolecular cytoskeletal elements, such as those employing small angle neutron scattering to determine the structural attributes of microtubules in solution, and those employing computational algorithms to quantitatively fit scattering cross-sections to various molecular structures, including the aberrant forms that result from the binding of vinca alkaloids and other antimitotic drugs. Also, a method was worked out to quantify changes in the mechanical strength of the cortical actin network formed in neutrophils when the latter are activated by chemoattractants or other extracellular ligands.

Laboratory of Mammalian Genes and Development. The LMGD employs advanced gene targeting and transgenic technologies to study genes that control specific stages of mouse development. Present interest is focused on the development of the central and peripheral nervous systems, on pituitary and thymus development, and on mechanisms of genomic imprinting. Significant advances have been made in the understanding of genes that regulate pituitary organogenesis, intestinal innervation, and T cell development. The laboratory also participates in collaborative studies aimed at generating mouse models of prevalent human genetic disorders, presently including neoplasias, obesity, renal malfunction, congenital heart diseases, intestinal aganglionosis, and ganglioside storage disorders.

One group in the LMGD studies the role of T cell antigen receptor (TCR) signal transduction in thymocyte development and selection. Recent work has involved the generation of mice lacking different subunits of the TCR complex by gene targeting. Reconstitution of these mutants with transgenes encoding signaling-proficient or -deficient proteins has allowed a systematic study of the function of individual TCR subunits in development

and mature T cell function. The results of these investigations reveal that the individual TCR signaling subunits perform specialized functions at different points in development. In addition, they show that the multisubunit nature of the TCR signaling is required for signal amplification, a property critical for selection of the T cell repertoire in the thymus.

A third group in the LMGD is investigating genomic imprinting, an unusual form of gene regulation in which expression of a gene is restricted to one allele dependent on the parental origin of the allele. At least 25 genes are known to be imprinted in mouse and humans. Disruptions in normal imprinting patterns are associated with developmental disorders and cancer. The group is focusing on imprinting of a cluster of genes on the distal end of mouse chromosome 7, has generated a contig and physical map of the region, and has identified novel imprinted genes in the cluster. One gene of particular interest is the mouse *Kvlqt1* homolog. In humans, mutations in *KVLQT1* are associated with long QT syndrome. Imprinting of mouse *Kvlqt1* is under strong developmental and tissue-specific control, explaining the interesting inheritance patterns of long QT. In addition, the group has generated transgenic mice that identify a control region upstream of the mouse *H19* gene responsible for imprinted regulation of several genes in the cluster.

Laboratory of Molecular Embryology. Members of this Laboratory (LME) are investigating the mechanisms that determine whether genes are active or inactive in the context of chromatin, specifically the interplay between chromosomal structure and specific transcription factors. Recent work has shown that newly replicated *Xenopus* DNA is rapidly complexed with histone proteins, but that specific transcription factors may still gain access to their cognate sites. Apparently, modification of histone proteins, together with the staged assembly of nucleosomes and the chromatin fiber, are important for facilitating access to the DNA while the chromosome is still immature. Workers in the Laboratory recently demonstrated that the subsequent targeted modification of the histone proteins by acetylation or deacetylation can reversibly control transcription factor access to chromatin subsequent to replication. The group also found that nucleosome mobility facilitates transcription factor access to chromatin. Linker histones can restrict transcription on chromatin templates independently of the mobility or modification of the core histones. The capacity of linker histones to repress transcription is reflected in their affinity constants for linker DNA. Thus, the precise organization of regulatory DNA elements into chromatin structures is important both for DNA replication and transcription *in vivo*. This work has also led to a new model for nucleosome structure, in which the winged helix domain of linker histones is asymmetrically incorporated into the nucleosome, and which explains the role of these histones in transcriptional activation. Workers in this group also established that transcription factor TFIIA-dependent recognition of the TATA box by its cognate binding protein, TBP, depends on dissociation of the amino tails of core histones and on the position of the TATA box within the nucleosome. Investigations into the chromatin environment of disease genes such as those causing Fragile X syndrome, an X-linked heritable form of mental retardation, have revealed that the expansion and methylation of CGG triplets characteristic of this disease gene cause its nucleosomes to become unusually stable.

Other members of the LME are studying Ran, a small GTPase that is required for nuclear transport and the regulation of mitosis. Among other findings, they have shown that *Xenopus* RanGAP1, a critical regulator of Ran, and RanBP2, a important nuclear pore protein, undergo conjugation with SUMO-1, which is the acronym for small ubiquitin-related modifier. This modification is catalyzed by Ubc9p, a protein that is essential for cell cycle progression and cyclin B proteolysis. This modification is likely to be important for regulating Ran's activity in both nuclear transport and cell cycle control. Further studies are under way on the Ran GTPase pathway and on the SUMO-1 conjugation pathway to understand their biochemistry and regulation.

A group within the LME is interested in tadpole metamorphosis, and especially in the mechanism by which the primitive tadpole gut is reorganized into the complex adult tract with stomach and intestines. This remarkable change, which involves selective cell death and proliferation, is entirely controlled by thyroid hormone (TH) through the TH receptor (TR), which heterodimerizes with 9-*cis* retinoic acid receptor (RXR). The group has characterized many TH-response genes in the intestine during this transition. Among them are genes encoding matrix metalloproteinases (MMPs). The group has obtained evidence to suggest that MMPs are involved in extracellular matrix (ECM) remodeling, which in turn influences cell behavior, especially larval epithelial apoptosis and adult cell proliferation. These workers have developed *in vitro* cell and organ culture systems, which has allowed them to investigate how these proteins regulate cell-cell and cell-ECM interactions and how the ECM influences cell fate. Their work has also demonstrated that TR/RXR can function within a chromatin context, that transcriptional activation leads to chromatin disruption, and that both TR and RXR are required to efficiently mediate the effects of T3 on both embryonic development and specific gene regulation.

Another group within LME is interested in how repressed chromatin states are established and inherited at the silent *HMR locus* in the yeast *Saccharomyces cerevisiae*. Silencing is mediated by a complex set of interactions between *cis*-acting elements and *trans*-acting factors, which result in the formation of repressed chromatin domains, which, once established, are epigenetically inherited. They are thus ideal for studying the structure of heterochromatin domains and the factors involved in their establishment and inheritance through multiple cycles of replication and cell division. These studies have identified a novel cell division cycle gene, *SAS10*, which also affects the inheritance of the repressed state, as well as mutations in gene products involved in DNA replication and cell cycle progression that affect the establishment and inheritance of the silent state. In conjunction with these genetic analyses, biochemical studies are in progress to characterize the structure of the heterochromatic domain, as well as to reconstitute it *in vitro* using novel yeast cell free extracts.

Members of the LME also investigate mechanisms used by eukaryotic cells to ensure proper chromosome transmission in mitosis. Their studies concentrate mainly around identification of new proteins crucial for chromosome structure and the characterization of a new family of nuclear proteins, the SMC family. The proteins of this group are found in the majority of cellular organisms and are indispensable for proper chromosome segregation in mitosis. The budding yeast has four *SMC* loci, all of which are essential for viability. Mammalian cells also have four different SMC proteins. The first goal of the ongoing studies is to elucidate the degree of specificity and redundancy among different types of SMC proteins in the same eukaryotic organism. We are approaching this problem through the functional dissection of four *SMC* genes and by searching biochemically and genetically for the interacting proteins in budding yeast. The first gene identified in the course of this study, *MCD1*, was found to encode an essential polypeptide functionally linking mitotic chromosome condensation and sister chromatid cohesion *in vivo*. The role played by SMC proteins in mitotic chromatin is also being assessed, and an *in vitro* assay for their biochemical activity is being developed. Another interest of this group is cell-cycle dependent regulation of the SMC proteins in the mammalian cell.

Laboratory of Molecular Genetics. Investigators in this laboratory (LMG) study the molecular and cellular basis of animal development and also seek to identify important evolutionary similarities in developmental pathways. Accordingly, one major interest is embryonic induction and axis specification in amphibia and another easily manipulated vertebrate, the zebrafish. Axis specification at the gastrula stage is regulated by the region named the Spemann organizer. Its function can be studied by analyzing genes that are specifically expressed in this area. LMG investigators have identified the homeobox gene *Xlim-1* as an early Spemann organizer-specific gene. An activated form of the *Xlim-1*-encoded protein can transform ectodermal cells into neural cells and mesodermal cells into muscle cells, while neural patterning requires the cooperation of Xlim-1 with the distinct transcription factor Xbra. Axis formation further requires the function of signaling molecules of the nodal family, members of which are being studied in the zebrafish. Two nodal-related factors, ndr1 and ndr2, are expressed in non-overlapping regions of the shield, the zebrafish equivalent of the organizer. In later development, ndr2 is expressed in asymmetric fashion along the left-right axis in the mesoderm and the brain, and is likely to be involved in establishing left-right asymmetry. In a third project, the role of Wnt signaling molecules in neural patterning has been studied. Certain members of the Wnt family, such as Wnt-1 and Wnt-3a, are capable of inducing neural crest markers in neural ectoderm, and the inhibition of Wnt signaling blocks neural crest formation in the whole embryo. Thus, Wnt factors are involved in the formation of the neural crest.

One of the vertebrate homologs (*Dlx3*) of the *Drosophila* homeotic gene *Distal-less* is also being studied. In mouse skin, *Dlx3* is expressed during terminal differentiation of epidermal cells, and is apparently an important factor in controlling this process, as shown by misexpression studies in transgenic mice. Dlx3 is a transcriptional activator protein, and presumably up-regulates the expression of a battery of target genes. One candidate target is the gene encoding the epidermal protein profilaggrin, which is activated in cells ectopically expressing *Dlx3* and which has a strong binding site for this homeoprotein in its regulatory region.

Another group in the LMG is concerned with a series of RNA processing steps that lie between the synthesis and translation of RNA, including polyadenylation, splicing, and transport. During these processes, RNA is usually complexed with proteins and organized into RNP particles. To study the function of nuclear pre-mRNA binding (hnRNP) proteins, these workers have undertaken a genetic analysis of three genes encoding hnRNP proteins. Two of the genes, *Hrb98DE* and *Hrb87F*, are non-essential, but double mutants show synthetic lethality. This is specific for combinations of mutations in the *Hrb* genes, and is not seen with other hnRNP proteins. In contrast, mutations in the third gene, caz, cause lethality, demonstrating that some hnRNP proteins are essential for viability. In other studies, a cytoplasmic RNA binding protein, NTS, was shown to be required for normal eye development and male and female fertility. Genetic studies implicate this protein in signal transduction during eye development.

The fruitfly *Drosophila* has also been used as a model for homeotic gene function. In the past year, LMG workers have continued to study genes that act as suppressors or enhancers of homeotic mutations. The group has also found that one-third of these genes also regulate an important signaling gene, *hedgehog*. Evidence was obtained that three such regulatory genes, *brahma*, *osa*, and *moira*, encode proteins that are conserved from yeast to mammals and are involved in regulating transcription of target genes through *cis*-regulatory response elements. The response elements for *brahma* regulation have been mapped in three target genes. Two of these *brahma* response elements require *osa* function, while the third does not.

Other LMG investigators have concentrated on RNase H, an enzyme that degrades the RNA strand in an RNA/DNA duplex, is involved in a number of critical cellular processes, including selection of the origin of DNA replication, plays a critical role in the retroviral life cycle, and participates in selective removal of RNAs when cells are treated with therapeutic oligonucleotides. Previous work in this lab has determined the three-dimensional structure of RNase H, a result of singular importance, given our current knowledge of retroviruses in human biology. In addition, the differences between cellular and retroviral RNases H has been exploited to inhibit retrovirus production, a potential means of controlling the spread of the HIV virus. Cellular RNases H of eukaryotes (from yeasts to humans) have been obtained and all contain a domain that interacts with duplex RNAs. Understanding of the regulation of these eukaryotic enzymes should be valuable in enhancing therapeutic oligonucleotide drug development.

LMG workers have established that bacterial global transcriptional responses to nutritional stress, such as amino acid and carbon deprivation, are mediated by the pyrophosphorylated nucleotides ppGpp and pppGpp. A genetic approach to this previously elusive mechanism is provided by their isolation of mutants of the transcriptional machinery (RNA polymerase) that compensate for the complete absence of (p)ppGpp. Of these, detailed studies on mutants of sigma-70 reveal that open complexes of RNA polymerase and DNA for certain promoters can be destabilized along with abortive initiation of RNA chains and promoter clearance. LMG investigators are now asking if lesions in these processes are also displayed by RNA polymerase core subunit mutants.

Replication of genetic information in the life cycle of the retroviruses involves a complex series of steps catalyzed by the virion-associated reverse transcriptase. LMG investigators are probing protein-nucleic acid interactions important for priming of HIV plus-strand synthesis by the polypurine tract primer and have identified residues in two major structural elements in reverse transcriptase that are essential for this event. They have also shown that the HIV-1 nucleocapsid protein functions as an accessory factor in reverse transcription, which increases the efficiency and specificity of viral DNA synthesis during minus-strand transfer and subsequent elongation of minus-strand DNA.

Another group of investigators studies genetic recombination and transcription termination in *E. coli*. The Integrase family is a group of related enzymes that recombine DNA molecules that carry specific nucleotide sequences. These workers wish to determine the amino acid and nucleotide residues that confer specificity on the protein-DNA interaction, and to learn how changes in specificity occur in nature. In a second project, this group is studying properties of RNA polymerase, the central enzyme of transcription. RNA polymerase is extremely processive: once it has initiated polymerization, it continues to elongate the transcript until it encounters a terminator. Members of the group have discovered nucleotide sequences that suppress termination. These sequences lie upstream of the terminators they suppress, and their RNA transcripts directly convert polymerase to a terminator-resistant form. Currently, an *in vitro* transcription system is being used to characterize the RNA-RNA polymerase interaction and to determine how polymerase is modified.

A new group explores how cell-cell interactions determine the pattern of neurogenesis in zebrafish embryos. These workers use a combination of cellular, molecular, genetic, and computational approaches to identify molecular mechanisms and model how cell-cell interactions lead to the emergence of a relatively simple pattern of early neurons in the neural plate. The lab is currently focusing on analyzing a number of zebrafish mutants with aberrant patterns of neurogenesis.

Another new group in the LMG is using the zebrafish to study vertebrate organogenesis, in particular the embryogenesis of the circulatory system. Using mutations with specific defects in the embryonic blood cells or blood vessels, as well as variety of experimental tools, these workers are attempting to elucidate the embryonic origins of vascular endothelial cells and of hematopoietic stem cells and to determine how these two lineages are related, and to understand how the specification and patterning of the developing vasculature is controlled.

Laboratory of Molecular Growth Regulation. One group in this laboratory (LMGR) works on gene regulation in the immune system, focusing on the role of two transcription factors, RXRβ and ICSBP. RXRβ, a member of

the large nuclear hormone receptor superfamily, heterodimerizes with other receptors and regulates ligand-dependent transcription. ICSBP, a member of the interferon regulatory factor (IRF) family, regulates expression of interferon and interferon-inducible genes. In an attempt to understand the mechanisms by which these factors regulate transcription, factors that interact with RXR heterodimers as well as ICSBP have been investigated. These workers found that RXR, in a heterodimer with RAR and bound to a specific DNA element, interacts with the histone acetylase PCAF as well as with the co-activators p300/CBP, which also have histone acetylase activity, in a ligand-dependent manner. Similarly, these histone acetylases are recruited by DNA that is bound to IRF-1 and IRF-2. Data from reporter analysis clearly showed that the histone acetylase recruitment leads to enhanced transcription by these transcription factors. Furthermore, increased expression of histone acetylase genes led to increased biological effects of ligands and cytokines in transfected cells. These results strongly indicate that binding of specific transcription factors alters the chromatin structure in the promoter by recruiting modifiers of histone acetylation. We also found that TFIIB, a component of the basal transcription complex, strongly interacts with RXR heterodimers, indicating that transcription factors interact with various nuclear factors at multiple surfaces. To elucidate the *in vivo* role of ICSBP, we have studied host defense in ICSBP-/- mice. Among many pathogens, ICSBP-/- mice are susceptible to *Toxoplasma gondii*, and die soon after infection. Analysis of cytokine gene expression revealed that ICSBP-/- mice fail to induce the master cytokine IL-12 in macrophages upon infection, which leads to a severe deficiency in interferon γ production. This leaves ICSBP-/- mice unprotected from the infectious agent. Our studies thus revealed a previously unidentified cytokine-interferon connection, in which ICSBP plays a critical role.

A group of LMGR workers continued their investigation of transcription by RNA polymerase III (pol III), the eukaryotic pol that synthesizes tRNAs, 5S rRNA, and other small RNAs necessary for cellular proliferation. Their studies included human Alu and adenovirus small RNA genes as model pol III transcription units. Alu sequences are repetitive elements that have been transposed to nearly one million sites in the human genome, sometimes causing insertional mutagenesis in humans. Viral infection and certain transforming proteins work in part through the pol III transcription apparatus, inducing Alu transcripts. LMGR workers have found that a major point of regulation of pol III transcription is through phosphorylation and dephosphorylation of the human La protein, a pol III transcription termination factor that was previously known as an autoantigen in patients suffering from systemic *lupus erythematosus* and other autoimmune disorders. Their studies have provided an important regulatory link between the transcription termination and reinitiation steps in the pol III transcription process.

Other LMGR staff are continuing to investigate the nature of origins of DNA replication in eukaryotic chromosomes, and the requirements for DNA replication and transcription at the earliest stages in animal development. In differentiated cells, replication has now been shown to begin at specific loci in which one or more high frequency initiation sites are surrounded by several low frequency sites. Replication origins are determined by specific DNA sequences acted upon by nuclear structure, chromatin organization and DNA methylation. Specific initiation sites are established during each G1-phase of the cell cycle and can be activated *in vitro* by soluble factors in frog eggs. Analysis of these events at the beginning of mouse development revealed that mTEAD-2 is one of the first transcription factors expressed in the mouse zygote, and can activate both promoters and enhancers in cleavage stage mouse embryos.

The LMGR's portfolio of projects also includes an investigation into the regulation of RNA polymerase II-mediated transcription, focusing on the interactions of the TATA-binding protein with other polypeptide components of the TFIID transcriptional complex. Previously, this group of workers had identified nine TATA-binding protein associated factors (TAFs) from *Drosophila* and cloned many of them. Some of the smaller polypeptides may mimic core histone structure, thus allowing the transcription complex to displace histones and gain access to the DNA. A central question in eukaryotic transcription is how transcription is initiated in a chromatin context. One important mechanism is thought to involve acetylation of histone tails. A characteristic feature of transcriptionally active chromatin is a predominance of hyperacetylated histones, whereas hypoacetylated histones accumulate within transcriptionally silent domains. Recently, these workers identified various histone acetyltransferases, namely PCAF, p300, CBP, hGCN5, ACTR, and TAF250, and are presently investigating how these acetyltransferases contribute to transcriptional activation.

One group in the LMGR is interested in the mechanisms of cellular senescence and its converse, cellular immortalization. Cultures of normal mammalian cells, when propagated *in vitro*, approach a crisis point at which the vast majority of cells undergo apoptosis or enter a stable state of cell cycle arrest termed replicative senescence. The number of population doublings required to reach crisis is strongly dependent on the initial differentiation state of the cells, and is influenced by a number of signal transduction pathways, particularly those involved in stress responses. This close tie between differentiation state and the rate at which cells

approach crisis strongly suggests that the initial pattern of gene expression, and likewise the propensity of that pattern to undergo cell cycle-dependent reprogramming, are critical determinants of replicative senescence. It is now widely accepted that such reprogramming is regulated by higher order chromatin structure, both at the level of *cis*-acting gene control elements and through the remodeling of large, epigenetically inherited, chromatin domains. Higher order chromatin structure is in turn modulated by histone acetyltransferases and deacetylases as well as putative chromatin structural proteins, e.g., members of the HP1 family, and other silencing factors whose roles are less well understood. This group of workers is currently cloning and analyzing the structure-function relationships of acetyltransferases and deacetylases, and using retrovirus vector systems to transduce normal mouse and human cells with wild-type and dominant negative forms of these gene products. It was recently revealed in such experiments, for example, that overexpression of the transcription factor P/CAF accelerates the approach to crisis in a manner dependent on its histone acetyltransferase activity. By manipulating the expression of this and other modulators of chromatin structure, especially in null backgrounds that can be generated in transgenic mouse model systems, new insights are being gained into the molecular mechanisms underlying cell proliferation, apoptosis, and senescence.

Laboratory of Physical and Structural Biology. This laboratory (LPSB) seeks to understand biological structures through the physical forces that animate them by measuring force as a function of separation between molecules from all classes of bio-matter. This "osmotic stress" strategy used for forces between molecules allows the conformation of molecules such as ionic channels to be changed by moving the parts of single molecules. These workers also observe the formation of ordered molecular assemblies ("liquid-crystals") of molecules such as collagen, DNA, or lipids, measuring their energies of assembly to realize the connection between molecular force, organization, and dynamics.

One group studies biomolecular and biophysical mechanisms of membrane transport through "mesoscopic" ion channels, channels with relatively large aqueous pores, up to several nanometers in diameter. By analyzing statistical properties of channel-mediated ion currents at the single-channel level, structure/function questions are addressed using original physicochemical approaches, such as graded osmotic response, "molecular Coulter counting", access resistance, and reversible ionization of the channel amino-acid residues. The group has also initiated studies on information transfer in voltage-dependent ion channels with special emphasis on Stochastic Resonance and the role of ambient noise in the biology and medicine of sensory transduction. These studies have led to the first experimental demonstration and theory of noise-facilitated information transfer at the molecular level: Stochastic Resonance in voltage-dependent ion channels. This widely discussed result is a breakthrough in our understanding how biological systems can benefit from ambient noise. It shows that stochastic resonance, i.e., noise-induced improvement in signal transduction, is an inherent property of all biochemical reactions whose statistics are exponentially dependent on external parameters.

Another group seeks to understand the mechanisms of biomolecular recognition and assembly and to determine the forces that organize proteins and regulate their stability and function. It works to develop theory and to measure interactions between helical macromolecules. There is special emphasis at present on collagen assembly with the intent to establish physical principles of collagen fibrillogenesis and collagen fiber stability. Abnormal collagen-collagen interactions are responsible for severe pathology of human development (*osteogenesis imperfecta*) and for disease complications (e.g., connective tissue failure in diabetes). Improved knowledge of forces is likely to lead to useful insights for solving more general problems such as protein folding. The group has developed new symmetry laws for helix-helix interactions, exact analytical expressions valid for any patterns of discrete, charged surface residues. This is the first major advance in the theory of helix interaction in decades. It shows how the pitch of helices as well as their charge distribution determine changes in helical pitch in assemblies compared to solution. It answers important questions about DNA structure, i.e., why the helical repeat is different in solution from the helical repeat seen in well-hydrated assemblies of DNA.

A third group examines the organization of molecular assemblies. Its current purview includes: DNA/protein complexes, DNA/lipid assemblies, DNA assemblies such as are seen in viral capsids and *in vitro*, lipid/water systems, telomeric DNA analogs, polypeptides and polysaccharides. In all these systems, the intermolecular forces or interaction energies are measured. A significant goal is to characterize and codify measured forces in such a way that they can be used in computation, an undertaking that is strengthened by a strong foundation in physical theory, in particular statistical mechanics applied to liquid-crystals and to complex fluids. A major advance has been the theoretical solution of the "vapor pressure paradox", clarifying the difference between the solution properties of large biological molecules and their behavior within the confines of a virus or cell. This is a fifty-year old puzzle as to why surfaces have such a strong effect on

molecular organization. The theory has already stimulated validating experiments, and is the center of a five-laboratory cooperative experimental undertaking.

Laboratories of the Scientific Director. In the Section on DNA Replication, Repair, and Mutagenesis, considerable progress was made on a prokaryotic, SOS-inducible mutagenesis system, of particular interest since the genes that promote mutagenesis in bacteria have been much more clearly defined than those in eukaryotes. In *E. coli*, the products of the *umuDC* genes allow the DNA replication machinery to synthesize through unrepaired sites of DNA damage (which would otherwise stall the polymerase). This translesion synthesis is, however, error-prone; mutagenesis may be the cost of continued survival. DNA polymerase III holoenzyme, RecA and the UmuDC proteins are all directly required for mutagenic translesion synthesis, but the mechanism by which the UmuDC and RecA proteins promote mutagenesis is unknown. Earlier work by this group has shown that *E. coli* UmuD protein must be proteolytically processed to its active form, UmuD', by RecA. Members of the Section also found that RecA and UmuD' specifically interact, and that RecA facilitates the binding of UmuD' to DNA, suggesting a mechanism by which the Umu proteins are targeted by RecA to lesions in DNA. During the past year, the crystal structure of UmuD' was determined, which suggested that the protein can form filaments that bind to the RecA nucleoprotein filament. Further evidence emerged that UmuD' has two forms, a "molecular dimer" and a "filament dimer." Some mutations in the UmuD' filament domain inhibit mutagenesis, but others, which may allow better contacts between UmuD' and RecA or UmuC, enhance mutagenesis. In other work during the past year, these workers studied a *umuDC*-like operon in a plasmid, finding this operon within a region of the plasmid that also encodes a putative reverse transcriptase. This result suggests that the mutagenic operon may be embedded within a mobile genetic element, explaining how the *umu*-like genes might have moved between bacterial plasmids and chromosomes. Other experiments this year were devoted to a search for Umu homologs in other organisms, and a homolog of UmuC was found in the yeast *S. cerevisiae*. Disruption of the gene encoding this homolog resulted in modest UV sensitivity, and therefore the gene was designated *RAD30*. *RAD30* normally participates in a novel post-replication, error-free repair pathway, which is dependent on *RAD6* and *RAD18*. Although *RAD30*'s activity is error-free, in contrast to the error-prone activity of *umuC*, both genes are inducible by UV light.

Another group in this Section has previously identified, cloned, and sequenced the components of a highly conserved DNA damage-specific, DNA binding protein (DDB) complex, which is induced to high levels of binding activity by treatment of cells with UV light. The DDB complex recognizes a specific UV-photoproduct (6-4 pyrimidine dimers); it is the first such damage-specific, damage-inducible DNA binding protein to be identified in mammalian cells, and its activity is absent from the cells of patients with the heritable DNA repair disorder, *xeroderma pigmentosum* (Group E). Components of the complex include 127 and 48 kDa proteins. During the past year, a careful nuclear fractionation study indicated that the DDB complex is translocated from a loose association within the nucleus to a tight association, apparently with UV-damaged DNA in chromatin. However, only a small fraction of the p127 protein is translocated to the chromatin, suggesting that DDB may have another cellular function in addition to its apparent recognition role in nucleotide excision repair (NER). A specific interaction between p127 and Replication Protein A (RPA) was also seen, further supporting the notion that DDB functions in the damage-recognition step of NER. However, in a study of three newly identified XP-E cell lines, it was found that while RPA enhances NER in these cells, it does so non-specifically in normal and in other repair-deficient cells as well. Finally, this group found that the p48 component of the DDB complex also moves to damaged chromatin after UV irradiation, but is degraded soon thereafter, suggesting that p48, which is required to promote binding of p127 to damaged DNA, is the regulatory subunit of DDB. Since microinjected DDB corrects the repair defect in XP-E cells but is entirely unnecessary for repair in an *in vitro* assay when the substrate is naked DNA, this group hypothesizes that DDB is required for the repair of chromatinized, but not naked, DNA. This notion is of considerable interest since little work has been done on the repair of DNA in chromatin; most reports have focused on the *in vitro* repair of naked DNA, as was the case with transcription until rather recently.

The Section on Growth Factors investigates the mechanism of action of nerve growth factor (NGF) and the other neurotrophins. These factors are responsible for the survival and differentiation of sympathetic and sensory neurons in the peripheral nervous system and certain tracts in the central nervous system, as well. This group has found that the neurotrophins exert their effects through a family of tyrosine kinase receptors called the Trks. Binding to these receptors initiates a number of parallel kinase cascades that lead to changes in the phosphorylation and, consequently, the function of key proteins in the cell. Using the PC12 cell model, which differentiates in culture in response to nerve growth factor, it has been found that NGF increases the uptake of calcium from the medium and the release of calcium from intracellular stores. These actions of NGF lead to a

change in the intracellular concentration of calcium. Such changes appear to be important in neuronal survival, neuronal protection, and synaptic functioning. Surprisingly, both the Trk receptor and a low-affinity receptor for NGF, called p75, support calcium uptake by the cells. Present studies are designed to provide an understanding of the details of calcium uptake supported by these separate NGF receptors. Differentiation of the cells with NGF causes a decrease in the receptors for the mitogen, epidermal growth factor (EGF). It has been suggested that this decrease is part of the mechanism by which NGF instructs the cells to stop dividing and differentiate. It has been found that the decrease in the receptor is transcriptional in nature, depends on the integrity of the Ras/Raf/MAP kinase pathway, and is accompanied by an increase in the new transcriptional inhibitor, GCF2. Present studies are designed to provide a description of the transcriptional regulation of this important receptor. Treatment of the cells with NGF causes the induction and the phosphorylation of the transcriptional activator, NGFI-B. This early response gene is involved in the transcription of certain steroid-metabolizing enzymes and pro-opiomelanocortin, and appears to participate in T-cell apoptosis. It has been found that the NGF-induced phosphorylation of a specific serine prevents the binding of NGFI-B to the NBRE, the first identified response element for NGFI-B. Present studies are designed to provide an explanation of how this inhibition participates in changing the transcriptional repertoire of neurons when they form synapses and, thus, become exposed to NGF from the post-synaptic cell.

Perinatology Research Branch. The PRB is pioneering the use of fetoscopy, in combination with color Doppler ultrasound, for the diagnosis of fetuses suspected of having congenital anomalies. Color Doppler has proven to be of great utility in the case of anomalies that lend themselves to surgical remediation (e.g., the placement of various shunts), and in evaluating congenital cardiovascular abnormalities, reporting a landmark study describing the first successful endoscopic surgery on a human fetus. In a case of twin reverse arterial perfusion syndrome, the umbilical cord of an acardiac twin was surgically interrupted using transabdominal thin-gauge fetoscopy, thus saving the life of the normal twin, a procedure that has now been applied in many more cases. Recently, these workers were able to relieve congenital urethral obstruction in two fetuses by endoscopically placing, for the first time, a transurethral shunt, and they demonstrated the feasibility of endoscopically devascularizing a chorioangioma.

Studies on the role of subclinical infection and cytokines in premature birth have revealed that more than half of patients with suspected cervical incompetence in the third trimester have positive amniotic fluid cultures. When attempts are made to suture the dilated cervix in the presence of this subclinical microbial invasion, the outcome is uniformly poor (rupture of the membranes, clinical chorioamnionitis, or pregnancy loss). It was also found that the concentration and/or biological activity of cytokines in the amniotic fluid of patients with a positive amniotic fluid culture are several-fold higher in pre-term than in term labor. Apparently, ascending microbial infection has a more protracted course in preterm labor, leading to a larger inoculum and more time for macrophage activation and the mounting of an inflammatory response. The elevation of cytokine levels in amniotic fluid can be used diagnostically, since assay of the cytokines can be carried out much more rapidly than culture of the organism. In particular, it was found that IL-6 determination has the highest sensitivity of any available test for the detection of microbial invasion of the amniotic cavity. Beyond mere association, recent evidence obtained in this lab provides strong support for the notion that macrophage-derived cytokines participate in the mechanisms responsible for preterm labor due to infection. Recently, a subgroup of patients was identified with infection of the chorioamniotic space, who may be better candidates for antibiotic treatment than patients with amniotic infection, which has proven difficult to treat with antibiotics. Also, it was established that the monocyte/neutrophil system is activated in preterm fetuses, that both term and preterm infants experience a decrease in the impedance of cerebral blood flow during delivery. The etiology of periventricular leukomalacia was investigated, which occurs in up to a quarter of preterm infants that weigh less than 1500 g at birth, and which often leads to cerebral palsy.

OVERVIEW OF THE YEAR'S INSTITUTIONAL ACHIEVEMENTS

During the past year, we continued to experience considerable growth in our sources of support for young scientists and physicians who are receiving advanced training in research. A number of new fellowships are greatly enhancing our ability to offer both postdoctoral and pre-doctoral training. Our main postdoctoral mechanism is now the NIH Intramural Research Training Award (IRTA), which is the domestic equivalent of our longstanding Visiting Fellow Program for postdoctoral candidates from abroad. Both the IRTA and Visiting Fellow Programs have a five-year duration; a flexible pay range exists for both Fellowships, and the period of eligibility for these Fellowships extends for up to five years of previous postdoctoral experience. We are also using the IRTA authority to provide stipends to pre-doctoral fellows. These include students formally working

toward a Ph.D. in a graduate school, but carrying out their thesis work in one of our labs; students who spend a "sabbatical" year in the Institute while in graduate or medical school; and students who have recently graduated from college but who wish to spend a year or two in one of our labs before proceeding to graduate or medical school. This post-baccalaureate "year-off" program has quickly become extremely popular, with a large number of superb applicants. We have also established a new technical training program under the IRTA authority; this permits graduates with a Bachelor's or Master's degree, but with little previous laboratory experience, to spend up to three years in one of our labs, thereby receiving a mix of formal and "hands-on" training as research assistants. We have also been very successful in identifying new donors of external stipends, including endowments by private industry and foundations. The NIH's Fogarty International Center has continued to support sabbatical visits by senior scientists from abroad. In addition, a number of medical students supported by the Howard Hughes Medical Research Institute worked in our laboratories during 1997; as noted previously, these are students who have taken a year out of medical school to engage in full-time laboratory research at the NIH. Our Summer Student Program was very successful during 1997, with 150 undergraduate, graduate, and medical students working in the Institute. This program has also become extremely competitive, with several thousand applicants for the 1997 summer session.

During the coming year, we shall establish a new program for foreign pre-doctoral students, analogous to the pre-doctoral IRTA program. "Pre-Visiting Fellows" who have completed formal course requirements leading to the Ph.D. will, with the permission of their graduate faculties, carry out part or all of their dissertation research in one of our laboratories.

The NIH tenure track assures tenure candidates that they will have sufficient independent resources to fulfill their potential for excellence in a setting of complete academic freedom; as opposed to a permanent government job, the concept of "tenure" is thus employed as a useful and familiar indicator of institutional commitment to independent research career development, as it is conceived in research-oriented universities. Currently, this Institute is supporting 30 investigators in its tenure track. Recently, we also implemented the concept of a Staff Scientist. Such scientists have highly sophisticated and specialized skills that are critical to the success, over the long-term, of a team of biomedical researchers. However, Staff Scientists are not allocated independent research resources, and they work on projects assigned by the Principal Investigator of the team to which they belong. Currently, we have 14 Staff Scientists in our ranks.

Peer review of intramural research, conducted by the Institute's Board of Scientific Counselors and *ad hoc* experts, continues to receive great emphasis, with rigorous three-day site visits to each Laboratory and Branch at four-year intervals (as well as a less detailed, twice yearly review of the Intramural Program in its entirety). During 1997, visits were made to the Laboratories of the Scientific Director, the Laboratory of Developmental and Molecular Immunity, and the Heritable Disorders Branch, with detailed critiques prepared as a result of these visits.

A number of laboratories are involved in various aspects of research on AIDS: NICHD investigators receiving special NIH "targeted" AIDS grants in 1997 included Drs. Juan Bonifacino, Hui Ge, Henry Levin, and Judith Levin. Drs. Richard Klausner and Tracey Rouault are receiving extensive support from the NIH's Office of Minority Health Research for an investigation of African hemosiderosis (which may also be present in the American black population), which will hopefully lead to an identification of the mechanism that underlies this disease, as well as to new approaches to therapy. The Institute's efforts in technology transfer were also very successful during the past year. Currently, more than 40 NICHD research advances are at some stage of patent/licensing development, with special emphasis on the new vaccines created by the Laboratory of Developmental and Molecular Immunity and the Laser Capture Microdissection Apparatus developed by Dr. Bonner.

Seminars hosted by the 18 Laboratories and Branches in this Program were numerous and well attended throughout the year, such that this Institute organized a relatively large fraction of the NIH's overall offering of intramural seminars and workshops. During the past year also, a number of major research conferences with a national and international attendance were organized by NICHD scientists, including: *Glycoconjugates and Matrix Molecules in Health and Disease*, organized by Dr. Harry Chen, Endocrinology and Reproduction Research Branch; *Cell and Molecular Biology of the Nucleus* (Dr. Mary Dasso), Laboratory of Molecular Embryology; *NIH Immunology Interest Group Retreat* (Dr. Lawrence Samelson), Cell Biology and Metabolism Branch; *Lim Proteins* (Dr. Igor Dawid), Laboratory of Molecular Genetics; *Pertussis in Adults* (Dr. John Robbins) Laboratory of Developmental and Molecular Immunity; *Signal Transduction in Health and Disease* (Dr. Arthur

Levine), Office of the Scientific Director; *Advanced Techniques in Structural Biology: Microscale Exploration of the Proteosome* (Dr. Alfred Yergey), Laboratory of Cellular and Molecular Biophysics; *Is SV40 a Human Pathogen?* (Dr. Arthur Levine), Office of the Scientific Director; *GNRH Neurons* (Dr. Kevin Catt), Endocrinology and Reproduction Research Branch; *Hermansky-Pudlak Syndrome* (Dr. William Gahl), Heritable Disorders Branch; *Development in a Social and Biological Context* (Dr. Stephen Suomi), Laboratory of Comparative Ethology; *Neuropeptides and Drug Design in Health and Disease* (Dr. Douglas Brenneman), Laboratory of Developmental Neurobiology; *Control of Neuronal Genes, Development, and Synaptic Plasticity by Neural Impulses* (Dr. Douglas Fields), Laboratory of Developmental Neurobiology; *Keystone Meeting on Functional Organization of the Nucleus* (Dr. Alan Wolffe), Laboratory of Molecular Embryology; *Nuclear Hormone Receptors and Development* (Dr. Yun Bo Shi), Laboratory of Molecular Embryology; *Fetal Behavioral Development* (Dr. Roberto Romero), Perinatology Research Branch; and *Children-in-Focus* (Dr. Marc Bornstein), Laboratory of Comparative Ethology.

As noted earlier, a recently initiated collaboration involves the Laboratory of Cellular and Molecular Biophysics, led by Dr. Joshua Zimmerberg, and the National Aeronautics and Space Administration (NASA). In this collaboration, a new technology developed at NASA, the "Bioreactor", is being integrated with Dr. Zimmerberg's new technology of three-dimensional histoculture. The goal of this collaboration is to develop *in vitro* culture systems that permit a faithful replica of biologic processes (cellular, intercellular, intra-tissue, and intra-organ) occurring *in vivo*. NASA is transferring significant funds over the next five years to the NICHD in support of this effort.

During the past few years, a new effort has been made across the NIH to develop "special interest" groups that transcend the usual Laboratory and Institute barriers and create a forum for scientific exchange among all scientists on the Bethesda campus who share a common scholarly interest. There are now more than 30 special interest groups meeting at regular intervals to exchange discipline-specific experimental approaches and concepts, and to provide an informal critique of preliminary data generated by the group's members. Among the groups organized and led by the NICHD are these: Cell Biology (Dr. Lippincott-Schwartz); Immunology (Dr. Samelson); Developmental Biology (Dr. Dawid); *Drosophila* (Dr. Haynes); Glia (Dr. Gallo); Mouse Development (Dr. Westphal); Nerve Growth Factor (Dr. Guroff); RNA (Dr. Haynes); Yeast (Dr. Hinnebusch); *Xenopus*/Zebrafish (Dr. Sargent); Acetyltransferase (Dr. Klein); Biophysics (Dr. Basser); Cell Cycle (Dr. Dasso); and Endocrinology (Dr. Chrousos). These "special interest" groups also constitute an extraordinary resource in that they are well positioned to provide advice to the NIH on recruitment, search committee members, peer reviewers, and trends in their area of research.

During the past year, NICHD scientists received a number of honors, awards, and other recognition. Dr. Marianne Bernard received the Young Investigator Award of the European Neuroendocrinology Society; Dr. Juan Bonifacino was plenary speaker and chair at the annual meeting of the American Society for Cell Biology; and Dr. Marc Bornstein received the Avrold-Gesell Prize of the Theodor-Hellbrügge Foundation. Dr. Kevin Catt received the Annual Award of the Israel Endocrine Society and was also recognized on his birthday with a Symposium on Signal Transduction held in his honor in Tel Aviv. This was a gala occasion, with many of the post-doctoral fellows trained by Dr. Catt throughout his long career speaking at this excellent symposium. Dr. Simon Chandler received the Wellcome Trust International Prize, and Dr. George Chrousos received the 1997 Endocrine Society Clinical Investigator Award, the 1997 Rhone-Poulenc Rorer Award for Outstanding Achievements in Clinical Investigation, and the 1997 Hans Selye Award for Outstanding Contributions to Stress Research. Dr. Chrousos also delivered the J. Lester Gavrilove Lecture at the Mt. Sinai School of Medicine. Dr. Gordon Cutler was the Ernst Sommer Memorial Lecturer at the Oregon Health Sciences University and delivered the Jacques-Raymond Ducharme Lecture at the University of Montreal (a major loss to this Institute occurred during the past year when Dr. Cutler retired from the NIH and moved to a position with the Eli Lilly Corporation). Dr. Maria Dufau delivered a plenary lecture at a celebration of the 50th Anniversary of the award of the Nobel Prize to Dr. Bernardo Houssay, and Dr. William Gahl delivered the first Owen Gardner Lecture at Penn State University. Dr. Richard Klausner delivered the Rabbi Shai Shaknai Memorial Prize Lecture at Hebrew University, and the Harvey Lecture at Rockefeller University. He was also the keynote speaker at the annual Scientific Symposium of the University of Pennsylvania Cancer Center; the Distinguished Lecturer in the Markey Graduate Program in Cellular and Molecular Medicine, Johns Hopkins University; and the Distinguished Lecturer at the Center for Cancer Prevention, Harvard School of Public Health.

Dr. Arthur Levine delivered the Green Lecture at the European Molecular Biology Laboratory and the Keynote Lecture at the Institute of Medical Science (Tokyo)-Institute for Molecular Biology and Genetics (Seoul) Annual

Symposium. Dr. Levine was also invited to be the NIH Advisor to the newly established Asia-Pacific Rim International Molecular Biology Network. Dr. Jennifer Lippincott-Schwartz delivered the annual High School Symposium Lecture of the American Society of Cell Biology, and Dr. Y. Peng Loh received the Marsden Fund Award from the Royal Society of New Zealand. Dr. Bai Lu received the Department of Physiology's Trophy Honor (Kobe University, Japan), and he was also asked to represent Chinese-American scientists at the White House ceremony honoring the Chinese President Jiang Zemin's official visit to the United States. Dr. Joan Marini delivered the Klaus Hummler Lecture at the Childrens Hospital of Philadelphia and Dr. Keiko Ozato was appointed to the Editorial Board of *Molecular and Cellular Biology*. Dr. John Robbins delivered the first Porter W. Anderson Lecture at the University of Rochester, the Ian Hardy Memorial Lecture at the College of Physicians and Surgeons (Columbia University), and the Harold L. Steward Lecture at the Uniformed Services University of the Health Sciences. Dr. Roberto Romero presented the Edith Potter Memorial Lecture at the Annual Meeting of the American College of Obstetricians and Gynecologists and he also received the Annual Research Excellence Award of the Society of Perinatal Obstetricians.

Dr. Stephen Suomi presented a keynote lecture at the Annual Meeting of the American Neuropsychiatric Association, the 1997 Psi Chi Lecture at York College, the keynote lecture at McMaster University Research Day, and the keynote lecture at the Inaugural Meeting of the Board on Behavioral, Cognitive, and Sensory Sciences, National Academy of Sciences. Dr. Heiner Westphal received the Presidential Meritorious Rank Award, and Dr. Alan P. Wolffe was the Marcel Piche Lecturer at McGill University; Chair of the Keystone Symposium on the Nucleus; Chair of the CIBA Foundation Symposium on Epigenetics; and keynote lecturer at the Annual Meeting of the Danish Biological Society. Dr. Wolffe was also newly appointed to the Editorial Boards of *Science, Biochemical Journal, Molecular and Cellular Biology, and Nucleic Acid Research*. Finally, Dr. David Wassarman, a first-year tenure-track investigator in the Cell Biology and Metabolism Branch, became the first NIH intramural scientist to receive a Presidential Early Career Award. Sixty of these highly prestigious awards are made by the White House each year to encourage young investigators from throughout the country in every area of science and engineering. David's work focuses on a genetic dissection of the elements of signal transduction and transcription regulation in *Drosophila*.

Many of the Institute's other investigators held Visiting Professorships during the year, served as chairpersons at major meetings, or held major editorial positions with a diversity of scientific journals.

The Fellows Award for Research Excellence (FARE) is offered to NIH post-doctoral Fellows on the basis of abstracts of their current research submitted competitively. In 1997, more than 700 NIH post-docs submitted such abstracts and I am delighted to report that our Institute had more winners in 1997 than any of the other Institutes, despite the fact that we do not have the largest program. (We submitted 12% of the total abstracts but had 18% of the total number of winners.) The 21 NICHD awardees were: Drs. Alan Agulnick, Jorge Blanco, Margaret Chamberlin, Roger Davenport, Giovanni DiCola, Jing Du, Hao Fan, Jules Feledy, Jonathan Gastel, Yi Geng, Jianhui Guo, Rodney King, Gopal Kundu, Nicoletta Landsberger, Edna Mancilla, Emanuel Normant, John Presley, Michael Sasner, Joanne Sloan-Lancaster, K.V. Venkatachalem, and Jiemin Wong. Each winner receives $1,000 toward travel expenses for any U.S. scientific meeting—in addition to whatever travel funds had already been allocated by the Lab Chief for FY 1997. It is also notable that the Japan Society for the Promotion of Science has developed a new fellowship program at the NIH for our Japanese post-doctoral Fellows. In 1997, NICHD post-docs who are citizens of Japan won 12 such awards, or more than a third of the total awards given at the NIH.

During the past year also, the NICHD developed an entirely new grant program ("K-22") for its senior post-doctoral fellows that will ease their transition once they leave the NIH to take up academic positions elsewhere. Each year, senior NIH post-docs who have competed successfully for this prestigious award, and who have been offered tenure-track positions in universities or research institutes, will be granted $125,000 a year for their first two faculty years, with these funds directed toward partial salary support and laboratory operating expenses. We continue to offer aid to our trainees in other ways: This year, the NIH established an undergraduate scholarship program in which financially disadvantaged college students with an interest in biomedical research will receive college scholarships from the NIH of up to $20,000 yearly. They will work in one of our laboratories during school vacations and will be committed to spend a year at the NIH following graduation for each year of undergraduate scholarship. However, this obligation may be deferred until the completion of their final degree. We also continue our very successful Loan Repayment Program for post-doctoral fellows who have outstanding tuition debt from medical or graduate school. This program offers up to $20,000 yearly to successful competitors who currently have a large tuition debt *vis-à-vis* their NIH stipend.

The Senior Biomedical Research Service is now in its second year. This is a new personnel system for the NIH's most distinguished scientists, with a pay scale that transcends that of the Civil Service. About 15% of all SBRS appointments at the NIH have been given to NICHD scientists—a disproportionately high figure with respect to our total number of tenured scientists. Thus, this Institute continues to mount an ambitious and highly regarded Intramural Program with a continuing increase in the quality and quantity of its scientific productivity, even as the rate of growth in research support has declined. Currently, the workforce in this Program comprises some 1,000 people, focused on 90 independent research groups within the 18 Laboratories and Branches. Recently, the Institute for Scientific Information (ISI), in its newsletter *Science Watch*, reported that the NICHD's Intramural Research Program is first among all the intramural programs of the National Institutes of Health in a ranking of the highest-impact institutions (world-wide) in molecular biology and genetics during the past five years, based on the average number of times their papers have been cited by other researchers. Furthermore, the Program's stature is well reflected not only in the honors and awards accruing to its senior investigators, but in the new resources allocated to the Program over the past several years by the NIH and the Congress.

Arthur S. Levine, M.D.
Scientific Director
National Institute of Child Health
and Human Development

CELL BIOLOGY AND METABOLISM BRANCH

Richard D. Klausner, M.D., Chief

The Cell Biology and Metabolism Branch (CBMB) pursues studies in a variety of areas of cell and regulatory biology, including the structure and dynamics of the internal organization of eukaryotic cells, the biology and pathophysiology of metal metabolism, prokaryotic and eukaryotic gene regulation, the structural and biochemical basis of immune cell activation, and the biochemistry and cell biology of tumor suppressor genes. A wide array of techniques are brought to bear upon the scientific problems of interest to the investigators. In particular, CBMB has outstanding facilities and capabilities in microscopy with emphasis on electron-microscopy, including cryo-immunogold-microscopy, and on light and fluorescence microscopy. A powerful confocal microscope enhances the capacity to study intracellular fine structure and dynamics. A specially designed microscope, coupled to a computer-based imaging system, has allowed the Branch to explore more powerful ways of analyzing light microscopy, differential interference microscopy, and fluorescence microscopy. In addition, there are microinjection facilities both for *Xenopus* oocytes and a state-of-the-art mammalian cell microinjector. Facilities for the growth and study of the genetically tractable plant *Arabidopsis* and for *Drosophila* genetics are in place. The Branch has continued its clinical interests in disorders of metal metabolism, which includes an ongoing, extensive study aimed at a clinical and molecular description of iron overload in Africans and African Americans. This project, funded largely by the NIH Minority Health Program, includes a major field study in Zimbabwe and Swaziland aimed at identifying pedigrees in which iron overload is observed.

REGULATION OF INTRACELLULAR IRON METABOLISM

One of the projects in the laboratory aims at understanding the mechanisms and regulation of dealing with nutritional stress, in particular the metabolism by eukaryotes of essential but toxic metals such as iron. The Section on Human Iron Metabolism, directed by ***Tracey Rouault***, studies the regulation of intracellular iron metabolism in human cells. The two major regulatory proteins involved in sensing iron levels, IRP1 and IRP2, have been cloned. In order to better understand their mode of regulation, iron-sulfur cluster assembly and disassembly is being studied. Enzymes of iron-sulfur cluster assembly have been cloned. Pulse-chase studies have been used to define the iron-dependent degradation of IRP2, and mutagenesis is being utilized to better characterize the iron-dependent degradation signals. Over-expressed and purified apoprotein is being used to crystallize apoprotein and to make co-crystals with the RNA ligand. Using homologous recombination and electroporation of embryonic stem cells, we have created mice that are heterozygous or homozygous for deficiency of IRP1 and/or IRP2. Our work contributes to greater understanding of the uptake, utilization, and regulation of iron distribution, processes that are important in human nutrition. Regulation of iron uptake and distribution is important in cells and in animals because iron is an indispensable cofactor in numerous proteins, but excess iron is highly toxic. The reaction of iron with oxygen products creates free radicals and oxidative damage, and these reactions are thought to be important in processes such as aging and carcinogenesis. We are working towards identifying the quality control mechanisms used by cells in the defense against oxidative damage catalyzed by iron; insight into mechanisms of iron-catalyzed oxidative damage could be important in improving the health of higher organisms. Similarly, insights into the mechanism of mammalian iron uptake may contribute to our understanding of refractory anemias. Our work in characterizing genes important in intestinal iron uptake may lead to development of diagnostic tests and treatment strategies for iron overload syndromes.

Cloning and Characterization of Enzymes Involved in Assembly of Iron-Sulfur Clusters in Cytosol and Mitochondria of Eukaryotic Cells. Enzymes homologous to those involved in the assembly of iron-sulfur clusters of bacteria have been cloned in yeast and in mammalian cells. The homologs of bacterial NifS and NifU genes are localized to the mitochondria or cytosol of cells using specific 5′ targeting signals. Alternative use of in-frame AUGs determines whether the form of human NifS expressed will contain or lack a mitochondrial targeting sequence. These gene products appear to coassemble with several other proteins to facilitate insertion of iron-sulfur clusters into target proteins. Yeast genetics and biochemical techniques are being used to fully characterize the molecular complex and to determine the mechanism of assembly of iron-sulfur clusters.

Iron-Dependent Degradation of IRP2 Involves Metal-Catalyzed Oxidation of the Protein, Followed by Ubiquitination and Proteasomal Degradation. Binding studies have shown that IRP2 binds to consensus IREs with high affinity and with a specificity similar to that of IRP1. Whereas the RNA binding activity of IRP1 is regulated by the reversible assembly and disassembly of an iron-sulfur cluster in an otherwise stable protein, absolute levels of IRP2 are markedly reduced by iron treatment in several different cell types, and the fall in protein levels can be accounted for by a rise in the rate of degradation of IRP2. IRP1 and IRP2 are highly homologous proteins with an overall sequence identity of 58%. A major distinguishing feature between IRP1 and IRP2 is the presence of an insertion of 73 amino acids in domain 1 of IRP2, which contains the information required to effect iron-dependent degradation of IRP2, and of a chimeric construct into which the degradation domain has been inserted. Site-directed mutagenesis has revealed that cysteine residues within the degradation domain are indispensable in iron-dependent degradation, and that IRP2 is subject to metal-catalyzed oxidation in the presence of iron. In cells, IRP2 is degraded by the proteasome; use of proteasome inhibitors has permitted us to characterize intermediates in the degradation pathway. We have determined that IRP2 destined to be degraded by the proteasome is oxidatively modified, as indicated by acquisition of carbonyl groups, and that the marked protein is selectively ubiquitinated. Specific recognition of oxidatively modified proteins by the ubiquitin-proteasome system may account for turnover of many other proteins in mammalian cells.

NMR Solution Structure of an IRE. In collaboration with Pardi and colleagues at the University of Colorado, Boulder, we have obtained a solution structure of the consensus IRE, along with an alternative RNA stem-loop that is bound with high affinity by IRP1 only. The six-membered loop of the IRE is highly structured; a base-pair is present between positions C1 and G5 of the loop, and in addition, A2 is stacked on G5. G3 is structurally mobile and is in an unusual *syn* conformation. Data obtained in SELEX studies support the notion that G3 is an important contact in the high-affinity binding of IREs by IRPs. The other position in the IRE that shows considerable structural mobility is the bulge C of the stem, which is thought to be another important site of RNA-protein interaction.

Other Transcripts that are Regulated by IRP1 and IRP2. We have recently shown that the transcript of mitochondrial aconitase contains a functional IRE. However, the range of regulation is less than that seen with the ferritin IRE, and the levels of mitochondrial aconitase in animals on a low iron diet fall only by a factor of two. An energetically favorable alternative structure is identified in computer-directed folding of the mitochondrial aconitase sequence, and we speculate that the IRE conformation may only be intermittently present, possibly accounting for the smaller degree of translational repression seen.

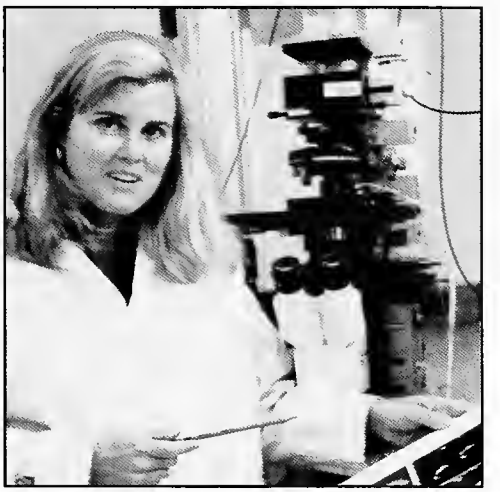
Physiological Role of IRPs. Gene targeting techniques have been used to generate loss of function mutations of IRP1 and IRP2 in mice. Mice lacking IRP1 are healthy, and iron uptake and metabolism appear to be normal. However, when cells of the knockout mice are treated with hydrogen peroxide, the cells lacking IRP1 do not repress ferritin biosynthesis, whereas cells containing IRP1 do, most likely because the iron-sulfur cluster of IRP1 disassembles. Homozygous knockouts of IRP2 have recently been created, and characterization of the phenotype is under way. Breeding of the mice should enable us to assess the phenotype of mice lacking the gene products of both IRP1 and IRP2.

Pathophysiology of Iron Overload in Humans. Hereditary hemochromatosis, a disease in which excessive intestinal iron absorption leads to parenchymal iron overload and tissue damage, is only seen in Caucasians; Africans and African Americans also develop iron overload, but genetic linkage studies have indicated that the genetic predisposition is not caused by the same disease gene as hereditary hemochromatosis. Since the disease gene of hereditary hemochromatosis, HFE, was recently cloned, we have evaluated African iron overload patients for mutations in HFE, and have determined that mutations in HFE are not important in African iron overload patients. Analysis of other candidate genes for iron overload is under way.

Iron Intoxication: Implications for Regulation of the Cell Cycle. Hemochromatosis is a human genetic disorder caused by excess uptake of iron from the diet, resulting in multi-organ failure and, often, liver cancer. Although the disease gene for this disorder was recently identified by positional cloning, the molecular basis of iron toxicity in cells remains largely unknown. We have investigated the toxic effects of excess iron accumulation in *S. cerevisiae* by expressing a dominant mutant allele of an iron-sensing transcription factor, *AFT1*. Expression of the dominant mutant allele *AFT1-1*^{up} results in high levels of ferrous iron uptake and is associated with slow growth in iron-rich media. *AFT1-1*^{up} strains transferred to iron-rich media accumulate as large, unbudded cells (85%). Flow cytometry revealed an accumulation of cells with 1N DNA content, consistent with a cell cycle arrest in G_1 or early S phase. The G_1 arrest is dependent on high affinity iron transport, since deletion of a yeast gene

important in iron uptake, *FET3,* will permit *AFT1-1^{up}* strains to grow in iron-rich media. The G_1 arrest appears not to require the activation of the *RAD9* DNA damage checkpoint. Rapid progression through G_1 is seen in strains expressing a hyperstable form of the G_1 cyclin CLN3 (*cln3-2*), and over-expression of *AFT1-1^{up}* in this strain fails to produce a cell cycle arrest in iron-rich media. These results suggested to us that the iron-mediated arrest could be bypassed in the presence of high levels of G_1 cyclin, prompting us to examine the regulation of these cyclins by iron.

Role of Cyclins in Cell Cycle Arrest. The G_1 arrest associated with iron intoxication is due to down- regulation of the G_1 cyclins Cln1 and Cln2 by iron. We demonstrated an iron- and Aft1-1^{up}-dependent decrease in the Cln2-associated kinase activity as well as in Cln1 and Cln2 protein levels. These changes in protein levels are not associated with any changes in mRNA levels, indicating that iron exerts its regulatory effects post-transcriptionally. Three mutant strains that poorly degrade the G1 cyclins, *cdc34-2*, *cdc53-1*, and *grr1*, were examined for the effects of *AFT1-1^{up}* and iron on Cln2 expression and cell cycle progression. These results indicated that defects in Cln2 degradation do not affect the iron-mediated down-regulation of the G1 cyclins or cell cycle arrest. We directly determined that the biosynthetic rate of Cln2 is reduced by the addition of iron in an *AFT1-1^{up}* strain, while there is no change in the rate of degradation of Cln2 under these conditions. These results indicate that iron regulates the expression of Cln2 (and presumably Cln1) by reducing the translation of the mRNA, a previously unreported mode of regulation of the G1 cyclins.



Tracey Rouault

Treatment of iron-arrested Aft1-1^{up} cells with mating pheromone demonstrated that the small fraction of cells with 2N DNA content is resistant to alpha factor, suggesting that a cell cycle arrest occurs in both G_1 and G_2. The G_2 block is exacerbated by deletion of the DNA repair genes *RAD1*, *RAD6*, *RAD52*, and *APN1*, and is dependent on the DNA damage checkpoint gene, *RAD9*. Expression of *AFT1-1^{up}* in strains deficient in DNA repair causes dramatic reductions in survival and markedly slows growth. Expression of *AFT1-1^{up}* is mildly recombinogenic and mutagenic. These data provide evidence that iron intoxication leads to DNA damage, which activates a cell cycle checkpoint that results in a cell cycle arrest in G_2. These data raise the intriguing possibility that the eukaryotic cell cycle is regulated by iron in a specific fashion.

GENE REGULATION IN RESPONSE TO ENVIRONMENTAL STRESS

Other research dealing with environmental stresses uses prokaryotes to study the genetics and biochemistry of the response to oxidative stress. The Unit on Environmental Gene Regulation, directed by ***Gisela Storz,*** studies the molecular mechanisms whereby organisms perceive an environmental signal and transduce this information into a change in gene expression. In one project, we are characterizing the *E. coli* and *S. cerevisiae* responses to oxidative stress, and in a second project, we are characterizing *Arabidopsis* mutants to elucidate how plant development is regulated by light.

Oxidative Stress Responses in Bacteria and Yeast. Reactive oxygen species ($O_2^{\bullet -}$, H_2O_2, and HO•) can lead to the damage of almost all cell components (DNA, lipid membranes, and proteins) and have been implicated as causative agents in several degenerative diseases. Most organisms have an adaptive response to defend against oxidants. Treatment of both bacterial and yeast cells with low doses of H_2O_2 results in the induction of distinct groups of proteins, the lower expression of other proteins, and enhanced resistance to killing by subsequent higher doses of H_2O_2. In bacterial cells, a key regulator of the defenses against H_2O_2 is the OxyR protein. OxyR appears to be both the sensor and transducer of the oxidative stress signal, since the oxidized but not the reduced form of the purified regulator can activate transcription *in vitro*. We are now examining the nature of the oxidative modification of OxyR. H_2O_2 treatment also induces the expression of *oxyS*, a unique, non-translated RNA, which has been shown to activate and repress as many as 40 genes in *E. coli*. The mechanism of oxyS action is now under investigation. Less is known about the induction of eukaryotic defenses against H_2O_2, but we have recently initiated studies to identify regulators of the *S. cerevisiae* response to oxidative stress.

Characterization of the Redox-Active Center of OxyR. We have undertaken both *in vivo* and *in vitro* approaches to further define the molecular details of OxyR oxidation and reduction. Denaturation, dialysis, and renaturation of OxyR does not affect the activity of the transcription factor, indicating that OxyR does not contain a metal ion or other prosthetic group. In contrast, a mutation in either of the two conserved cysteine residues (C199 and C208) in OxyR diminishes its ability to sense hydrogen peroxide. Mass spectrometry measurements and thiol-disulfide quantitation assays on the purified protein showed that the C199 and C208 residues are in a disulfide-bond form in the oxidized protein and in a dithiol form in the reduced protein. These findings suggest that OxyR activation and deactivation is a consequence of C199-C208 disulfide bond formation and reduction. To determine how the oxidized protein is reduced in the cell, we generated a set of strains having defects in either one of the two major disulfide-reduction systems; the glutathione/glutathione reductase pathway and the thioredoxin/thioredoxin reductase pathway. We found that the OxyR reduction is blocked in strains lacking GSH or glutaredoxin 1 (Grx1). In addition, *in vitro* transcription experiments showed that purified Grx1 indeed catalyzes OxyR reduction by GSH. By measuring the equilibrium constant of OxyR reduction, the redox potential of OxyR was determined to be -185 mV, a value that ensures OxyR is reduced in the absence of stress. We also found that the gene encoding Grx1 is itself regulated by OxyR, implying that the OxyR response is auto-regulated. Taken together, our results provide a clear example of redox-signaling through disulfide bond formation and reduction.

Regulation by the *oxyS* RNA. We are continuing our studies of the novel, 109 nucleotide RNA which is induced by H_2O_2. Previously, we found that the *oxyS* RNA acts as a pleiotropic regulator, leading to increased and decreased expression of multiple genes, and as an anti-mutator, protecting cells from spontaneous and chemically induced mutagenesis. Eight targets of *oxyS* regulation were identified, including *fhlA*, which encodes a transcriptional activator, and *rpoS*, which encodes the σs subunit of RNA polymerase. To gain insights into the mechanisms of *oxyS* action, we examined *oxyS* repression of *fhlA* and *rpoS*. Interestingly, deletion analysis showed that different domains of the RNA are required for the regulation of the two genes, suggesting that *oxyS* regulates different genes by different mechanisms. *oxyS* probably prevents *fhlA* translation by pairing with mRNA sequences overlapping the Shine-Dalgarno sequence, thereby blocking ribosome binding. *oxyS* repression of *rpoS* translation is dependent on the *hfq*-encoded RNA binding protein HF-I, and mutational and biochemical studies showed that HF-I binds to the *oxyS* RNA. We propose the following model to explain how *oxyS* inhibits *rpoS* translation by titrating HF-I binding. In the absence of other regulators, a secondary structure encompassing the *rpoS* Shine-Dalgarno sequence interferes with ribosome binding. HF-I recognizes and binds to the CUAAAG sequence that is paired with the Shine-Dalgarno sequence. This binding releases the *rpoS* mRNA for translation. When *oxyS* is induced by oxidative stress, the small RNA competes with HF-I, displacing it from the *rpoS* CUAAAG sequence, thus allowing reformation of the *rpoS* secondary structure and resulting in *rpoS* repression.

Larry Samelson and Gigi Storz

Regulators of the Yeast Response to Oxidative Stress. Relatively little is known about the eukaryotic defenses against oxidative damage. Therefore, we have also begun to examine the *S. cerevisiae* response to H_2O_2. To gain an overview of the known regulators that are important for protection against H_2O_2, we constructed isogenic strains carrying mutations in *YAP1*, *YAP2*, *SKN7*, *MSN2*, *MSN4*, *MAC1*, and *AFT1*. We are comparing the viability of these mutant strains under different stress conditions, and are examining the induction of antioxidant genes in the mutant strains. These studies indicate that the yAP1 and Skn7 regulators are most critical for resistance to H_2O_2. We have also begun to use the mutant backgrounds to isolate suppressor mutations in additional regulators and additional components of the oxidative stress signal transduction pathways.

Blue Light Regulation in *Arabidopsis*. The normal development of plants depends on light, and spectral studies have shown that plants undergo morphological changes in response red/far-red, blue, and UV light. For example, dark-grown plants have extremely elongated hypocotyls (stems) compared with seedlings grown under red or blue light. At present, the mechanisms by which the light signals are transduced into changes in plant growth and in morphology are largely unknown.

Characterization of *hy4* and *hy5* Mutants. In previous studies, we took advantage of the light-effect on hypocotyl length to screen for *Arabidopsis* mutants that have elongated hypocotyls under blue light. The 24 independent mutants we isolated fall into two complementation groups corresponding to the *HY4* and *HY5* genes. Since *HY4* is thought to encode the blue light photoreceptor, we characterized the 21 *hy4* mutants. One of these shows extremely interesting genetic behavior; the strain exhibits a wild-type phenotype in one generation and a mutant phenotype in the next. Since we have not been able to detect a molecular defect at the *HY4* locus, we propose that this line has an epigenetic mutation, the first such mutation to be identified in *Arabidopsis*. These studies of the *hy4* mutants were complemented by subtractive screens between wild-type, *hy4*, and *hy5* cDNA libraries to identify genes that are differentially expressed between the wild-type and mutant seedlings. Using this approach, we isolated two clones encoding novel cDNAs, which we are now characterizing.

REGULATION OF THE FATE OF NEWLY SYNTHESIZED PROTEINS IN THE CENTRAL VACUOLAR SYSTEM

The Section on Intracellular Protein Trafficking, headed by ***Juan Bonifacino***, investigates the molecular mechanisms that determine the localization and sorting of integral membrane proteins within the endocytic and secretory pathways. The main objectives of this project are the identification and characterization of signals involved in protein sorting within the endosomal-lysosomal system and of recognition molecules that bind to the signals. The interior of the cell is organized into a diverse array of membrane-bound compartments. Each of these compartments is characterized by the presence of a specific set of resident proteins. The localization of integral membrane proteins to the various compartments of the endocytic and secretory pathways is mediated by information encoded within the structure of the proteins. In some cases, targeting information is contained within short, linear sequences of amino acid residues that act as specific sorting signals. The signals are recognized by receptor-like molecules that are the primary effectors of protein sorting. In other cases, the targeting information is a manifestation of a global physical-chemical property of the proteins, such as a tendency to partition into membranes with a particular lipid composition or to form transport-incompetent aggregates in certain intracellular environments. Over the past year, our group has conducted studies on both types of mechanisms. Most of our work has focused on tyrosine-based sorting signals involved in internalization from the cell surface and in lysosomal targeting, and on adaptor complexes that function as receptor-like recognition molecules for such signals. We have also continued our studies on the role of the aggregation state of proteins on their sorting in post-Golgi compartments of the secretory pathway.

Role of Clathrin-Associated Adaptor Complexes in Sorting Integral Membrane Proteins to the Endosomal-Lysosomal System. Many integral membrane proteins contain within their cytosolic tails tyrosine-based sorting signals that conform to the tetrapeptide motif YXXØ, where Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic side chain (i.e., leucine, isoleucine, phenylalanine, methionine, valine). These signals mediate various sorting processes, including internalization from the cell surface and lysosomal targeting. Our laboratory had previously demonstrated that the medium chains, $\mu 1$ and $\mu 2$, of the clathrin-associated adaptor complexes, AP-1 and AP-2, function as recognition molecules for tyrosine-based sorting signals. AP-1 localizes to the trans-Golgi network and likely mediates targeting of newly synthesized proteins to lysosomes, whereas AP-2 localizes to the plasma membrane and mediates protein internalization from the cell surface. Both $\mu 1$ and $\mu 2$ exhibit characteristic preferences for residues neighboring the critical tyrosine residue of the signals. (i.e., residues at the X and Ø positions). These distinct preferences, as well as the location of the signal within the cytosolic tail, determine whether a particular tyrosine-based motif will function as a lysosomal targeting signal or internalization signal.

Juan Bonifacino and Jennifer Lippincott-Schwartz

AP-3: a Novel Adaptor Complex Involved in the Recognition of Tyrosine-Based Sorting Signals. While the involvement of tyrosine-based sorting signals in lysosomal targeting and internalization has been known for many years, recent studies have shown that these signals mediate various other sorting processes, including transport from early to late endosomes, delivery to specialized endosomal/lysosomal organelles such as pigment granules

and MHC class II antigen-processing compartments, and targeting to the basolateral surface of polarized epithelial cells. The finding that tyrosine-based sorting signals are involved in numerous sorting processes led us to hypothesize that there would be other adaptor complexes, in addition to AP-1 and AP-2, capable of recognizing tyrosine-based sorting signals. Indeed, searches of expressed sequence tag databases revealed the existence of several novel proteins related to subunits of AP-1 and AP-2. Based on this information, we isolated full-length cDNAs encoding these novel proteins. The cDNAs were sequenced and antibodies were raised to the predicted protein sequences. These reagents were then used to characterize novel adaptor complexes.

An antibody to a novel homolog (σ3) of the small chains σ1 and σ2 of AP-1 and AP-2, respectively, was found to immunoprecipitate from fibroblasts a complex of four proteins with molecular masses of 21 kDa, 47 kDa, 140 kDa, and 160 kDa. These proteins were identified using antibodies to putative adaptor subunit homologs, and were named σ3, μ3, β3A-adaptin, and δ-adaptin, respectively. The novel complex was named AP-3. The subunits of AP-3 were found to be expressed in all mammalian cells examined; homologs of these proteins were also identified in a wide variety of eukaryotic organisms, including yeast, worms, flies, and plants, suggesting that the AP-3 complex plays a general role in cellular physiology. Proteolytic digestion experiments demonstrated that the large subunits of AP-3, β3A- and δ-adaptin, are organized into "head", "hinge", and "ear" domains, similar to the analogous subunits of AP-1 and AP-2. β3A-adaptin is heavily phosphorylated on serine residues. Analyses using the yeast two-hybrid system demonstrated that μ3, like μ1 and μ2, binds to tyrosine-based sorting signals with characteristic specificity. Immunofluorescence microscopy experiments using antibodies to various AP-3 subunits revealed that the complex is associated with early endosomal structures that contain the transferrin receptor. No significant co-localization was observed with markers of the trans-Golgi network or late endosomes. Treatment with the fungal metabolite Brefeldin A causes rapid dissociation of AP-3 from membranes into the cytosol, suggesting that the complex may be regulated by the small GTP-binding protein ARF1. These observations suggest that the AP-3 complex is an adaptor complex involved in protein sorting in endosomal compartments, possibly for transport from early to late endosomes or from early endosomes to the basolateral plasma membrane of polarized epithelial cells.

Altered Expression of δ-Adaptin Leads to Defective Pigment Granule Biogenesis in the *Drosophila* Eye Color Mutant *garnet*. An important clue to the function of the AP-3 complex came from the identification of a homolog of δ-adaptin in *Drosophila melanogaster*. Based on partial sequence information obtained from DNA databases, we isolated a full-length cDNA encoding *Drosophila* δ-adaptin and found that transcripts specified by this cDNA are altered in mutants of the *garnet* eye pigmentation locus. Histological analyses by light and electron microscopy revealed that *garnet* mutant flies have reduced numbers of eye pigment granules, which correlates with decreased levels of both pteridine (red) and ommachrome (brown) pigments. Thus, the eye pigmentation defect in *Drosophila garnet* mutants can be attributed to compromised function of the AP-3 complex in intracellular transport processes required for biogenesis of pigment granules. Since pigment granules have characteristics of late endosomal-lysosomal organelles, the defect in the *garnet* mutants is consistent with the idea that AP-3 plays a role in sorting proteins from early to late endosomes. The connection of AP-3 with pigment granule biogenesis in flies illustrates the value of *Drosophila* as a model organism to study the function of adaptor complexes in protein trafficking.

Sequence Preferences for Interaction of Tyrosine-Based Sorting Signals with Adaptor μ Chains Determined by Combinatorial Methods. A possible explanation for the involvement of tyrosine-based sorting signals in several sorting processes is that different signals are recognized with characteristic fine specificity by the various adaptor complexes. Our identification of the adaptor medium chains (μ1, μ2, and μ3) as the recognition molecules for tyrosine-based sorting signals has allowed analysis of this problem at a molecular level. In order to establish the sequence preferences for interaction, we used a yeast two-hybrid system to screen combinatorial peptide libraries with μ1, μ2, and μ3 as selector molecules. This approach revealed that each μ chain has a characteristic preference for residues around the critical tyrosine. In general, μ1, μ2, and μ3 were found to prefer neutral, basic, and acidic residues, respectively, at the X positions of the YXXØ motif. The μ2 chain exhibits the broadest specificity, consistent with the fact that most tyrosine-based signals function as internalization signals whereas only a limited subset of signals mediate intracellular sorting events.

Cloning of the Gene Encoding Murine μ2: Gene Organization, Alternative Splicing, and Chromosomal Assignment. To begin a genetic analysis of the physiological role of the adaptor medium chains in mammals, we have cloned the genomic DNA encoding murine μ2. The μ2 gene is approximately 8.5 kb long and is organized into 12 exons and 11 introns. Two transcripts are generated by alternative splicing of exon 5, a mini-exon of only six nucleotides. Proteins encoded by both transcripts are capable of interacting with tyrosine-based sorting signals,

suggesting that they are functionally equivalent. The $\mu 2$ gene is localized in the proximal region of mouse chromosome 16, which is analogous to the proximal region of human chromosome 3. The $\mu 2$ gene is the first medium chain gene for which this information is available. Isolation of the $\mu 2$ gene should facilitate future mutational analyses of $\mu 2$ function in mice.

Bipartite Domain Organization of $\mu 1$ and $\mu 2$. The adaptor medium chains $\mu 1$ and $\mu 2$ have been implicated in two types of interaction: assembly with the β1 and β2 chains of AP-1 and AP-2, respectively, and recognition of tyrosine-based sorting signals. We performed a structure-function analysis of the $\mu 1$ and $\mu 2$ chains aimed at identifying regions of the molecules that are responsible for each of the two interactions. Analyses using the yeast two-hybrid system suggest that $\mu 1$ and $\mu 2$ have a bipartite structure, with the amino-terminal one-third (residues 1-145 of $\mu 1$ and $\mu 2$) being involved in assembly with the β chains, and the carboxy-terminal two-thirds (residues 147-423 of $\mu 1$ and 164-435 of $\mu 2$) binding to tyrosine-based sorting signals. Proteolytic digestion experiments support such a bipartite structure for $\mu 2$ by demonstrating that the amino-terminal one-third is resistant to digestion by high concentrations of trypsin. These observations suggest a model in which the amino-terminal one-third is embedded within the adaptor core via interaction with the β chain, whereas the carboxy-terminal two-thirds project outwards from the core, placing this region in a position to interact with tyrosine-based sorting signals.

Aggregation as a Determinant of Protein Sorting in Post-Golgi Compartments. All the studies described so far have dealt with signal-mediated protein sorting. However, we have also performed studies on the role of aggregation in the sorting of the endopeptidase furin in post-Golgi compartments. Furin is a type-I integral membrane protein that is predominantly localized to the trans-Golgi network (TGN) at steady state. A few years ago, we demonstrated that furin has a relatively short half-life due to its targeting to lysosomes for degradation. We have now found that the lysosomal targeting of furin correlates with aggregation of the protein within the TGN. In addition, we demonstrated that both aggregation and lysosomal targeting are mediated by the luminal domain of furin, strongly suggesting that the two phenomena are linked. These observations illustrate another mechanism of protein sorting, which does not directly depend on sorting signals but is mediated by changes in the global physical-chemical properties of the proteins.

LOCALIZATION AND DYNAMICS OF INTRACELLULAR ORGANELLES

The Unit on Organelle Biology, led by ***Jennifer Lippincott-Schwartz***, seeks to understand organizational principles underlying organelle structure and disassembly/reassembly pathways, and to characterize molecular components involved in these processes. Over the past year, this group has focused on the cell cycle dynamics of three organelles: the nuclear envelope, the endoplasmic reticulum (ER), and the Golgi complex. Protein chimeras containing the green fluorescent protein (GFP) have been generated that localize within each of these compartments or which move between them. They were used with state-of-the-art fluorescent imaging techniques, including confocal time-lapse imaging, deconvolution and photobleaching technology, to describe the disassembly and reassembly of the nuclear envelope and the Golgi complex during mitosis, the lateral mobility of ER resident proteins, and the characteristics of secretory membrane traffic.

Nuclear Envelope Disassembly/Reassembly During Mitosis. The mechanisms by which membrane proteins are localized to the inner nuclear membrane and their fate during mitosis are not well understood. To address this question, we analyzed the dynamics of the inner nuclear membrane protein, lamin B receptor, fused to green fluorescent protein (LBR-GFP), during the cell cycle in living cells. In interphase, the majority of LBR-GFP was found to be immobilized within nuclear envelope (NE) membranes, whereas a subpopulation within ER membranes is entirely mobile and diffuses rapidly ($D = 0.41 \pm 0.1\ \mu m^2 sec^{-1}$). High resolution confocal time-lapse imaging revealed that after NE disassembly during mitosis, LBR-GFP redistributes from NE membranes into the interconnected ER, exhibiting the same high mobility and diffusion constant as in interphase ER membranes. Furthermore, LBR-GFP rapidly diffused across the cell within the membrane network defined by the ER, indicating that the integrity of the ER is maintained during mitosis, with little or no fragmentation or vesiculation. Nuclear membrane reassembly at the end of mitosis coincides with immobilization of LBR-GFP at contact sites between ER elements and chromatin. LBR-GFP-containing ER membranes then wrap around chromatin over the course of two to three minutes, quickly and efficiently compartmentalizing nuclear material. Expansion of the NE follows over the course of 30 to 80 minutes. Thus, changes in lateral mobility of LBR-GFP within the ER/NE membrane system serve to mediate its retention in the inner nuclear membrane during interphase. Such changes, rather than vesiculation mechanisms, also underlie its redistributions during NE disassembly and reformation in mitosis.

ER-to-Golgi Transport Visualized in Living Cells. All newly synthesized proteins that leave the ER are funneled through the Golgi complex before being sorted to different final destinations, but the nature of the membrane transport intermediates that mediate ER-to-Golgi passage is not fully understood. Two basic models have been proposed. In one, proteins move through a long-lived "intermediate compartment", which continually packages and buds vesicles for transport into the Golgi complex. In another, cargo-containing vesicles budding from the ER fuse to form large, pleiomorphic structures, which themselves translocate to and fuse with the Golgi. Both models envisage passage of proteins through tubulovesicular "intermediate" structures before reaching the Golgi complex. A key distinguishing feature between these models is that the first views these structures as stable compartments with resident proteins and functions, while the second envisions them as transient elements, which themselves ferry protein to the Golgi complex. The proper characterization of these intermediate structures, therefore, has important implications for understanding secretory membrane traffic and Golgi biogenesis.

To evaluate these two models, we attached green fluorescent protein to the cytoplasmic tail of G protein from the ts045 mutant strain of vesicular stomatitis virus, (VSVG-GFP), in order to visualize ER-to-Golgi membrane traffic in living cells. Upon exit from the ER, fluorescent chimeric membrane proteins become concentrated in peripheral tubulovesicular structures enriched in ßCOP, previously described as the intermediate compartment. By tracking these proteins, the intermediate compartment was found to translocate to and fuse with the Golgi complex, rather than to act as a stable structure for packaging of Golgi-targeted transport vesicles. Translocation occurs without loss of accumulated chimeric proteins, at rates of 1μm/sec, is sensitive to nocodazole and requires the microtubule minus-end-directed motor complex of dynein/dynactin. These data indicate that ER-to-Golgi transport intermediates are transient structures, which utilize microtubules to transport their concentrated cargo to the Golgi complex.

Golgi-to-Plasma Membrane Transport Visualized in Living Cells. The nature of membrane transport intermediates conveying proteins and lipid from the Golgi complex to the plasma membrane (PM) was also studied using VSVG-GFP in live cells. Passage of VSVG through the Golgi complex was quantitated in single cells by measuring changes in Golgi intensity versus time after shift to permissive temperature. Within 30 to 40 minutes of temperature shift, ER-derived VSVG-GFP completely redistributes into the Golgi complex. Export of VSVG-GFP from the Golgi is slower than ER export and transport to the Golgi complex, requiring over 90 minutes to be complete. Time-lapse confocal and video microscopy revealed that VSVG-GFP is exported from the Golgi complex in large, irregularly shaped structures, which bud as a whole from the *trans*-face of the Golgi complex. These structures are depleted of Golgi markers and do not contain COPI or the adaptor complexes AP1 and AP2. Their morphologies are dynamic, and they often stretch into tubular shapes as they move at velocities of up to 2μm/sec along microtubules. Depolymerization of microtubules with nocodazole arrests movement of the post-Golgi intermediates, but delivery of VSVG-GFP to the plasma membrane still occurs, presumably due to direct fusion of the intermediates with nearby plasma membrane sites. Disruption of the actin cytoskeleton has no effect on the budding or translocation of the intermediates. Taken together, these data indicate that large, complex membranous intermediates, rather then small vesicles, mediate traffic from the Golgi complex to the cell surface. Microtubules are essential for translocation of these intermediates, but not for their generation or fusion with the plasma membrane.

Golgi Disassembly and Reassembly in Mitotic Cells. The Golgi apparatus undergoes a profound reorganization during mitosis in many cell types and is widely assumed to break up into thousands of small vesicles scattered throughout the cytoplasm, which remain inactive until the end of mitosis, when they actively fuse to regenerate Golgi stacks. Ultrastructural studies of mitotic cells have supported this view by showing Golgi proteins in vesicle clusters. However, Golgi proteins have also been observed in the continuous membranes of the ER during mitosis, raising the possibility that localization of these proteins is not restricted to static vesicles. To investigate this possibility, we followed the fate of Golgi membranes in mitosis in living cells by time-lapse confocal microscopy, using the Golgi enzyme galactosyltransferase, GalTase, tagged with GFP. The Golgi complex normally appears as a set of flattened cisternae, but completely disassembles at the end of prophase, with Golgi resident proteins such as GalTase-GFP dispersing into membranes distributed throughout the cell. The dispersed mitotic membranes containing Golgi proteins also contain ER markers, suggesting that Golgi membrane proteins redistribute into the ER system during mitosis rather than into membrane fragments or vesicles. Consistent with this view, measurements of the diffusional mobility of GalTase-GFP in mitotic cells using photobleaching techniques revealed these proteins to be extensively mobile and capable of diffusing rapidly from one region of the cell to another. Furthermore, the value of the diffusion coefficient, D, of GalTase-GFP is identical to that in ER

membranes of interphase cells. These findings strongly suggest, therefore, that Golgi proteins in mitotic cells are not localized solely in static vesicles, but are diffusing rapidly within the interconnected ER membrane system.

Golgi Tubule Traffic and the Effects of Brefeldin A Visualized in Living Cells. The Golgi complex is engaged in both secretory and retrograde membrane traffic. To gain insight into the morphological dynamics of this organelle *in vivo*, we used GFP-Golgi protein chimeras in time-lapse confocal microscopy experiments. In untreated cells, membrane tubules are a ubiquitous, prominent feature of Golgi morphology, serving both to interconnect adjacent Golgi elements and to carry membrane outward along microtubules after detaching from stable Golgi structures. Brefeldin A (BFA) treatment, which reversibly disassembles the Golgi complex, accentuates tubule formation without tubule detachment. A tubule network extending throughout the cytoplasm was found to be quickly generated and to persist for 5 to 10 minutes, until it rapidly empties Golgi contents into the ER (within 15 to 30 seconds). Both lipid and protein leave the Golgi at similar rapid rates, with no Golgi structure remaining, indicating that Golgi membranes do not simply mix but are absorbed into the ER in BFA-treated cells. The directionality of redistribution implies that Golgi membranes are at a higher free energy state than ER membranes. Analysis of the kinetics of redistribution, using computer algorithms for diffusive transport, points to a mechanism that is analogous to wetting or adsorptive phenomena in which a tension-driven membrane flow supplements diffusive transfer of Golgi membrane into the ER. Such nonselective, flow-assisted transport of Golgi membranes into the ER suggests that mechanisms that regulate retrograde tubule formation and detachment from the Golgi complex are integral to the existence and maintenance of this organelle.

Diffusional Mobility of Misfolded Proteins in the ER. The ER contains a meshwork of interacting resident proteins that provide a specialized environment for folding and maturation of membrane and secretory proteins. ER-specific chaperones and folding enzymes repetitively associate with folding intermediates to facilitate proper folding and assembly into transport-competent protein complexes, while proteins incapable of folding correctly are retained in the ER. Despite knowledge of types of interactions between misfolded proteins and lumenal membrane components, the manner in which such interactions affect the dynamic properties of misfolded proteins, including their diffusional mobility in the plane of the ER membrane and ability to egress from the ER, is not known.

To study interactions that might underlie retention of misfolded proteins in the ER, we used fluorescence recovery after photobleaching (FRAP) experiments to examine the diffusional mobility of the temperature-sensitive misfolding mutant of VSVG protein tagged with green fluorescent protein (VSVG-GFP). At its permissive temperature of 32°C, VSVG folds properly and is capable of being exported from the ER, while at its nonpermissive temperature of 40°C it misfolds and is retained in the ER. FRAP of VSVG-GFP in ER membranes at 32°C revealed a rapidly moving chimera ($D = 0.44\ \mu m^2/sec$) with little or no immobile fraction. Upon shift to nonpermissive temperature, no significant change in D or mobile fraction of VSVG-GFP was observed, with the chimera diffusing rapidly within the ER despite being misfolded. The diffusional mobility of VSVG-GFP at 40°C does not vary with length of time at nonpermissive temperature nor does it depend on the overall level of expression of the chimera. It is comparable to that of other membrane proteins residing in the ER, including the ß subunit of the signal sequence receptor and lamin B receptor. Conditions that prevent VSVG-GFP's dissociation from BiP, including ATP depletion or expression of a BiP ATPase mutant, however, reduce the mobility of VSVG-GFP in ER membranes, with greater than 30% of the chimera immobilized at 40°C. These conditions have no effect on the mobility of membrane proteins in the ER, which do not misfold or interact with BiP. These results suggest that interactions between misfolded VSVG-GFP and lumenal components such as BiP in unperturbed cells do not affect the diffusional mobility of VSVG-GFP in ER membranes. Thus, ER retention of misfolded VSVG does not depend on protein immobilization or aggregation.

SIGNAL TRANSDUCTION

The Section on Lymphocyte Signaling, headed by ***Lawrence Samelson***, explores the biochemical basis of T cell activation in both the human and murine systems. This group has been particularly interested in the engagement of the multi-component antigen receptor in T cells (TCR), which results in the rapid activation of one or more tyrosine kinase pathways.

The T Cell Receptor Tyrosine Kinase(s). Identifying and characterizing the kinase(s) responsible for tyrosine phosphorylation of the TCR and of other cellular substrates has been a major goal of this Section for many years. A critical tyrosine kinase activated by the T cell receptor is the ZAP-70 protein, which is tyrosine-phosphorylated

and associates with the TCR upon activation. In previous years, we have studied the interaction of this kinase with the TCR, defined the sites of phosphorylation on this enzyme and their regulatory effect, and have purified a large amount of this kinase to study its enzymatic properties. In this past year, we have continued to study this critical protein tyrosine kinase, making use of a new and valuable reagent, a T cell line lacking ZAP-70, which was prepared by our collaborators at the Mayo Clinic. These investigators mutagenized the Jurkat T cell line and selected variants that fail to demonstrate signs of T-cell-receptor-mediated signaling, such as the induction of intracellular tyrosine phosphorylation and the elevation of intracellular calcium. Their evaluation of this variant indicated an absence of ZAP-70 protein. In our collaborative studies, we were able to demonstrate that re-expression of ZAP-70 using mammalian expression vectors reconstitutes all the deficiencies of the cells lacking ZAP-70. In contrast, overexpression of a defective form of ZAP-70 in the kinase-deficient cell line fails to reconstitute T cell function. TCR engagement leads to elaboration of lymphokines and to development of effector functions. In addition, engagement of this receptor can lead to activation of a "death" pathway, which if not inhibited, leads to apoptosis. This pathway is now known to be mediated in T cells by up-regulation of the Fas ligand (FasL), which upon engagement by the Fas molecule leads to T cell death. The ZAP-70-deficient cells fail to undergo TCR-induced apoptosis. Further analysis revealed that these cells fail to up-regulate FasL upon TCR engagement. Reconstitution of these cells with wild-type ZAP-70 protein restores this response. However, expression of forms of ZAP-70 having a mutation either in the ATP-binding domain or in the kinase domain activation loop, both known to inhibit kinase function, fails to reconstitute TCR-mediated FasL up-regulation or apoptosis.

In the past year, an entirely different approach has led to new insights into the function of the ZAP-70 protein tyrosine kinase. All our earlier work and the vast majority of published studies have focused on the biochemical properties of this protein. This year, we took a cell-biological approach to analyze ZAP-70. To this end, we created a chimeric molecule in which ZAP-70 is directly coupled to the green fluorescent protein (GFP). This construction results in a protein with the enzymatic properties of ZAP-70, but with a tag that can be used to track its intracellular localization. When expressed in COS cells, the ZAP-70 GFP chimera was found to be localized in the cytoplasm, and surprisingly, in the nucleus (see below). Pharmacologic activation of tyrosine phosphorylation in these cells by addition of pervanadate led to a migration of the ZAP-70 GFP protein to the cell membrane. This phenotype is enhanced when the chimeric protein is co-expressed with the activated Lck protein tyrosine kinase, a means to enhance activation of ZAP-70 function. Using advanced video imaging techniques, we were able to follow this process in live cells over time. These experiments supported the earlier studies in fixed cells. ZAP-70 moves to the membrane upon activation, and this process is enhanced kinetically and quantitatively by addition of the Lck enzyme. Additional experiments indicated the importance of the phosphorylation and activation state of ZAP-70 to this process, as a mutant inactive form of the kinase coupled to GFP showed little movement to the membrane upon pharmacologic activation. The addition of Lck has a less dramatic effect on the translocation of this mutant ZAP-70 GFP molecule. We also reconstituted the above-described ZAP-70-deficient cell line with the ZAP-70 GFP chimera. These cells demonstrate normal TCR-mediated increases in tyrosine phosphorylation. Cytoplasmic and nuclear localization of the enzyme was observed. TCR engagement leads to re-distribution of some of the chimeric protein to the cell membrane. These studies have provided important new information about the dynamic properties of this critical protein.

Localization of ZAP-70 to the nucleus was revealed in these studies in both fibroblasts and T cells. The presence of nuclear protein was found to be independent of the level of protein expression, indicating that this localization is not an artifact of over-expression. These observations also led us to evaluate whether there is nuclear expression of ZAP-70 in non-transfected cells. These studies required affinity purification of our antibodies to the protein. With them we were able to confirm the presence of ZAP-70 in the nucleus of normal Jurkat T cells. A biochemical analysis was then performed to supplement these microscopic findings. ZAP-70 was found by protein blotting in isolated Jurkat nuclei. Moreover TCR ligation led to enhanced tyrosine phosphorylation of this nuclear fraction of the enzyme, suggesting that this fraction is active. At this point, we have little understanding of the function of this subset of ZAP-70 molecules. Studies are underway to explore this question.

The Tyrosine Phosphorylation Pathway in T Cells. In previous studies we have demonstrated that activation of the TCR results in phosphorylation of the TCRζ chain and of multiple intracellular substrates. The identity of the other substrates that are tyrosine-phosphorylated upon TCR activation has been a major question for this Section for some time. One substrate of TCR-mediated tyrosine kinase activation is the protooncogene product $p120^{cbl}$ (Cbl), which is rapidly tyrosine phosphorylated upon T cell activation. Our studies and those of others

demonstrated that this protein is a complex adaptor molecule, which binds to a large number of critical signaling molecules.

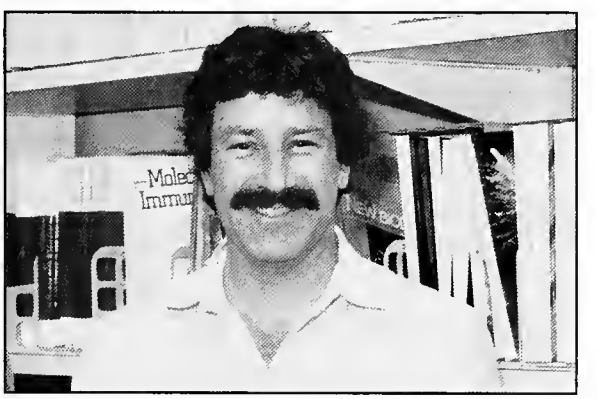
The function of Cbl was also studied in RBL-2H3 cells, which can be activated by engagement of the FcεRI. We demonstrated that in this system Cbl is also tyrosine phosphorylated upon receptor engagement. Using recombinant vaccinia viruses to overexpress various protein tyrosine kinases, we demonstrated that Syk, a kinase closely related to ZAP-70 is responsible for Cbl tyrosine phosphorylation in RBL-2H3 cells. We found that Cbl and Syk form a complex in RBL-2H3 cells. The tandem SH2 domain of Syk is sufficient to bind to residues 1-655 of Cbl. Interestingly, we also found that the Cbl found within this complex is not tyrosine phosphorylated. This result suggests that there may be functional significance to the interaction of Cbl with the Syk kinase.

To further analyze the effect of Cbl overexpression, we overexpressed both it and Syk in the RBL-2H3 cells, and evaluated the status of Syk activity. Overexpression of Cbl led to a dose-dependent inhibition of Syk tyrosine phosphorylation and of Syk tyrosine kinase activity. A truncation mutant of Cbl that binds to Syk inhibits Syk activity, but a shorter mutant (1-480) fails to block it. Interestingly, a mutant form of Cbl that is transforming, isolated from the 70Z pre-B cell line, also fails to inhibit Syk function. Overexpression of Cbl has this effect on Syk by virtue of its ability to block Syk association with the FcεRI receptor, which becomes tyrosine phosphorylated on the gamma chain despite the overexpression of Cbl. The effect of the inhibition of Syk association with the receptor and its failure of activation is a global inhibition of intracellular tyrosine phosphorylation. Furthermore, the overexpression of those forms of Cbl that block Syk activation results in inhibition of mast cell function, as measured by the block in receptor-mediated serotonin release. Much work has been directed at understanding the function of the Cbl protein in a number of receptor systems in which activation of tyrosine kinases is critical. It is clear from multiple studies that Cbl serves as a docking protein for a number of molecules involved in signaling cascades. The study reviewed here indicates another possible function of the protein as a regulator of kinase localization and function.

THE VHL TUMOR SUPPRESSOR GENE

A project led by ***Richard Klausner***, who heads the Section on Organelle and Receptor Structure and Function, is directed at understanding a novel tumor suppressor gene, VHL, and the biochemistry and cell biology of the VHL gene product.

VHL is Part of a Multi-Component Protein Complex. We previously showed that the VHL protein (pVHL) forms a stable complex with two proteins called elongin B and elongin C, two factors that were originally identified as part of a heterotrimeric complex that stabilizes and activates the transcription elongation factor, elongin A. Elongin B has high homology to a ubiquitin domain protein, and elongin C has significant homology to a recently described family of proteins called SKP. In yeast, SKP proteins link Cdc53 to targets for ubiquitin-mediated protein degradation. We have continued to search for other components of the pVHL protein complex. By making a column containing recombinant VBC complexes, we purified from cell cytosol a protein that specifically associates with the VBC complex. This protein was purified, microsequenced, and identified as a member of a newly described family of proteins called cullins. The human protein that we identified as part of the VBC complex is called Hs-CUL-2. There are six identified human cullins, and they are all related to a yeast protein, Cdc53. CUL-1 is the closest relative (by sequence homology) to Cdc53, and CUL-2 is closely related to CUL-1. The VHL CUL-2 interaction is specific for CUL-2, and no *in vitro* association is seen with other members of the cullin family. In addition, VHL will not bind to CUL-2 without assembling with elongin-B and C. Thus, almost 70% of naturally occurring germline mutations in VHL disrupt specifically the ability to assemble with B, C, and CUL-2. Our evidence suggests that all the endogenous VHL present in the cell is associated with B, C, and CUL-2. We do not yet know whether all CUL-2 present in the cell is bound to VBC. In *C. elegans*, null mutations in the CUL-2 homolog, CUL-1, result in hyperplasia in all tissues and are required for cell cycle exit. We have begun mapping the interaction site on



Richard Klausner

CUL-2 for the VBC complex. The cullins all share a high degree of homology in their carboxy-termini but are quite divergent in their amino-termini. We have been able to show that all the information for the CUL-2 VBC interaction is contained within the first 50 amino acids of CUL-2. We have not been able to identify a short-sequence motif required for this interaction, but rather a region of approximately 40 amino acids appears to be required in the CUL-2 sequence.

VHL and the Control of the Cell Cycle. In order to assess whether the expression or loss of expression of VHL affects the cell cycle, we have established a series of matched renal cell carcinoma cell lines that either lack any functional VHL or into which wild-type or mutant VHL has been reintroduced. We have observed these stable cell lines in terms of their *in vitro* behavior and compared cell lines that express between two and fifteen times the normal endogenous level of VHL. No differences in the phenotypes observed can be linked with these widely differing levels of VHL protein expression. VHL expression has no affect on the movement through the cell cycle or the rate of proliferation of cells in 10% serum. However, when cells are serum-starved, those that lack functional VHL continue to proliferate and fail to quiesce. This is a characteristic of many cancer cells. In the complete absence of serum, VHL^- cells will proliferate for several rounds and then undergo apoptotic cell death. In marked contrast to this phenotype, reintroduction of wild-type VHL into these VHL^- renal cell carcinoma cells results in a dramatic cell cycle arrest within 48 hours of serum deprivation. These quiescent cells survive for weeks and do not undergo cell death in response to the removal of growth factors. If cells are placed in serum-free medium supplemented with insulin, transferrin, and selenium, the VHL^- cells proliferate and do not die. In contrast, under these growth conditions the reconstituted VHL wild-type expressing cells are arrested and appear to be in G_0. Thus, we have established a role for VHL in responding to growth factor deprivation with exit from the cell cycle into G_0. The operational definition of the cells being in G_0 is the fact that the cells will re-enter the cycle in a synchronized fashion upon refeeding but only after a delay of 18 to 24 hours. As a control for these studies, HeLa cells were examined *in vitro*. These cells have the same expected phenotype of cancer cells in that they do not quiesce upon serum deprivation. These cells have a normal VHL gene and can be shown to express VHL protein. When exogenous VHL was introduced and overexpressed in these cells, there was no affect on the response to serum deprivation. Thus, we conclude that in the renal cell carcinoma lines the loss of VHL entirely explains the common cancer phenotype of an altered ability to sense growth factors and a reduced requirement for growth factors for cell cycling. In these cells, reintroduction of VHL is sufficient to correct this phenotype.

We have established several biochemical correlations for the VHL-dependent serum deprivation control of quiescence. In cells lacking functional VHL, reduction of serum results in no alteration in the level of cyclin-dependent kinase inhibitors such as p27 and p57. In contrast, reintroduction of VHL results in the ability of these cells to accumulate high levels of p27 in response to serum deprivation. The accumulation of p27 is entirely explained by stabilization of an otherwise rapidly turned over protein. We are intrigued, considering the assembly of VHL with CUL-2, that, in yeast, Cdc53 (a homolog of CUL-2) is associated with the regulated and targeted degradation of the yeast p27 homolog, CIP-1.

Defective Placental Vasculogenesis in VHL-Deficient Mice. The VHL gene was disrupted by targeted homologous recombination in murine embryonic stem cells, and a mouse line containing an inactive VHL allele was generated. While heterozygous VHL (+/-) mice appeared phenotypically normal, VHL -/- mice died *in utero* at 10.5 to 12.5 days of gestation (E10.5 to E12.5). Homozygous VHL -/- embryos appeared to develop normally until E9.5 to E10.5, when placental dysgenesis developed. Embryonic vasculogenesis of the placenta failed to occur in VHL -/- mice, and hemorrhagic lesions developed in the placenta. Subsequent hemorrhage in VHL -/- embryos caused necrosis and death. These results indicate that VHL expression is critical for normal extraembryonic vascular development.

Post-Transcriptional Regulation of Vascular Endothelial Growth Factor mRNA by VHL. VHL was stably introduced into a variety of human renal cell carcinoma cell lines by retroviral transduction. Matched cell lines expressing either wild-type or mutant VHL were compared with the parental cell line expressing only the retroviral vector. All the paired cell lines grew at equal rates in 10% serum as well as in soft agar. Despite this, the reintroduction of wild-type VHL resulted in a dramatic decrease in tumorigenicity for some of the cell lines when they were injected subcutaneously into nude mice. Examination of the tumor sites revealed a marked lack of stromal cell proliferation and vasculature in the small tumors seen in mice injected with cells containing wild-type VHL. This suggested the possibility that VHL expression inhibits the profuse vascular reaction that accompanies renal cell carcinomas and other tumors associated with the VHL syndrome. Indeed, the introduction of wild-type VHL results in a profound inhibition in the production and secretion of the vascular endothelial growth factor, VEGF. This was seen at both the protein and RNA level. Analysis of both transcription initiation

and elongation demonstrated that neither of these is the locus for the control of mRNA levels but rather that mRNA levels are altered through the stabilization or destabilization of VEGF mRNA. VHL mutant renal cell carcinoma lines constitutively express high levels of VEGF mRNA and VEGF protein. Reintroduction of VHL suppresses expression by destabilizing the VEGF mRNA. In the presence of wild-type VHL, VEGF can be induced by serum deprivation via stabilization of the message and accumulation of VEGF levels comparable to those seen in the parental VHL mutant cell lines. Thus, VHL allows the cells to sense serum deprivation in order to regulate the expression of this critical gene, which is likely to be involved in tumorigenicity and metastasis of renal cell carcinoma and other VHL-associated tumors.

VHL Intracellular Localization. To further understand the function of the VHL protein, we studied the interactions and trafficking of a pVHL fusion to the green fluorescent protein (GFP). Like wild-type pVHL, the hybrid protein, VHL-GFP, binds to elongin B and C, and regulates VEGF mRNA levels. VHL-GFP was found to be localized both in the cytoplasm and in the nucleus of all cell lines studied. Heterokaryon formation demonstrated that VHL-GFP shuttles between the nucleus and the cytoplasm. Inhibition of transcription by actinomycin D and 5,6-dichlorobenzimidazole riboside results in nuclear accumulation of VHL-GFP. We show here that pVHL is a nuclear/cytoplasmic trafficking protein, which accumulates in the nucleus when transcription is arrested. To our knowledge, this constitutes a novel trafficking pattern for a cellular protein.

TRANSCRIPTIONAL REGULATION

The Unit on Developmental Transcription Regulation, headed by ***David Wassarman***, is undertaking the genetic analysis of RNA polymerase II transcriptional factors in *Drosophila*. Transcriptional regulation is a basic process that occurs in all eukaryotic organisms. Mutations that affect the level, timing, or spatial distribution of transcription can cause severe developmental abnormalities. Since the transcriptional machinery displays a high degree of evolutionary conservation, an understanding of the factors and mechanisms that regulate transcription in *Drosophila* should be easily extrapolated to humans.

Genetic Screens Sensitive to Transcription Levels in *Drosophila*. To study transcriptional regulation *in vivo*, we have taken advantage of a signal transduction pathway that specifies the fate of R7 photoreceptors in the developing *Drosophila* eye. This pathway is mediated, in part, by the Sevenless (Sev) receptor tyrosine kinase and the Ras GTPase. Mutations in the *sev* gene inactivate this pathway, resulting in the development of R7 precursor cells as cone cells. In contrast, ectopic expression of constitutively active forms of *Sev* (*SevS*11) or *Ras* (*RasV*12) in cone cell precursors, using enhancer sequences from the *sev* gene and promoter sequences from the *hsp*70 or from the *sev* genes, (*SevS*11 or *sev-RasV*12, respectively), leads to their conversion to the R7 photoreceptor fate. The presence of extra R7 cells in the adult eye disrupts the normal packing of cells and produces an externally visible rough eye. A key feature of this system is that the severity of the rough eye phenotype is proportional to the level of expression from the *sev*-driven transgenes (see below). Thus, we have created a situation in which small changes in transcription levels can be easily detected as changes in the severity of the rough eye. This sensitized genetic background was used to screen for mutations that, when heterozygous, cause a suppression of the *sev-RasV*12-induced rough eye phenotype. We have isolated mutations at a number of loci, which by genetic criteria may act as regulators of *sev* transcription. Included in this group are mutations in two of the eight *Drosophila* TAFs, TAF60 and TAF110. Molecular characterization of the other loci is in progress. One of these loci, *SR*3-4A, has been narrowed down to a 30 kb region that contains five lethal genes, including DEAF-1, a known transcriptional activator. Another locus, *SR*3-5, maps to the same region as the TAF250 gene and displays genetic interactions that are similar to TAF60 and TAF110 mutations. We are currently sequencing the TAF250 gene from several *SR*3-5 alleles.

David Wassarman

To identify additional loci that regulate *sev* transcription, we have initiated a dominant modifier screen similar to the one described above. This screen is based on the observation that expression of both *sev-RasV*12 and

SevS11 in a single fly causes synthetic lethality (i.e., the flies die prior to eclosion). The synthetic lethality is suppressed by TAF60 or TAF110 loss-of-function mutations, suggesting that a decrease in the level of transcription from the *sev*-driven transgenes allows development to proceed more normally. This hypothesis is supported by the fact that *sev-RasV12* transgenic lines that express low levels of RasV12 (see below) are not synthetic lethal in combination with SevS11. We have screened mutations in known transcriptional activators and in approximately 1,000 lethal P-element lines, and have identified numerous dominant suppressors of the synthetic lethality, including RNA polymerase II subunits, histone deacetylase (*RPD3*), and trithorax group genes. These results indicate that screens of this type will lead to the identification of numerous *trans*-acting factors that regulate Pol II transcription. We plan to continue our genetic screens for genes that affect *sev* transcription. This includes completing the P-element screen and initiating an EMS screen. In addition, we will continue our molecular characterization of the *SR3-5* and *SR3-4A* alleles that were isolated as suppressors of the *sev-Ras1V12* rough eye phenotype and which display genetic interactions indicating that they encode transcriptional activators.

Quantitation of Transcription Levels *in Vivo*. The screens described above are not only sensitive to mutations in genes that affect transcription but also to genes that affect Ras1 signal transduction. Furthermore, mutations in transcriptional activators may exert their effect on the transgenes and/or endogenous genes required for Sev/Ras1 signal transduction. Therefore, we have developed a molecular assay to differentiate between these possibilities. Using RNase protection analysis, we are able to quantitate the steady state level of both the transgenic and endogenous forms of sev and Ras1 mRNAs expressed in the heads of flies that carry wild-type or mutant chromosomes.

This assay was used to determine that the level of *sev-Ras1V12* expression in five independent transgenic lines varies over a three-fold range. Genomic Southern analysis indicates that the difference in expression level is due to position effect, in the case of lines on the second chromosome (CR1, CR2, CR3), and a difference in the number of P-element insertions, in the case of lines on the third chromosome (TR4 and T2B). Lines with the highest level of expression (CR2, TR4, and T2B) are synthetic lethal in combination with *SevS11*, while lines with lower levels of expression (CR3 and CR1) are viable in combination with *SevS11*. This supports the hypothesis that the synthetic lethal phenotype directly correlates with the expression level of *sev-Ras1V12*.

We then used this assay to determine whether mutations that suppress the synthetic lethality affect the level of transcription from the *sev*-driven transgenes. Preliminary results indicate that mutations in TAF110, SR3-5, the 140 kD subunit of Pol II, and in some trithorax group genes reduce the expression level of *sev-Ras1V12*, while mutations in RPD3 have the opposite effect.

Identification of *sev* Enhancer Binding Proteins. The screens described above rely on the *sev* enhancer sequences to misexpress genes during *Drosophila* development. Therefore, to understand how factors, such as TAFs, are functioning in this context, we would like to identify the transcription factors that bind to the *sev* enhancer. Previous studies have defined a 475-bp enhancer fragment that confers correct expression of the *sev* gene. Dnase I footprinting of this fragment with extracts derived from fly heads reveals six protected regions. We are using a yeast one-hybrid screen to identify these DNA binding factors. Using mRNA isolated from fly heads, we are constructing a cDNA library to be used as the expression vector in the screen. In addition, we are constructing reporter plasmids containing portions of the *sev* enhancer. Screening this library in yeast should identify factors that activate transcription from particular *sev* enhancer sequences. After completion of the constructs, we will screen the adult head cDNA library with a number of reporter vectors containing different fragments from the *sev* enhancer. Isolated genes will be tested *in vitro* for their ability to bind to the appropriate enhancer fragment and for binding to TAFs. Reverse genetic approaches will be used to isolate mutations in these factors and determine their *in vivo* functions.

Identification of Gene Targets for TAFs. We are interested in how general factors such as TAFs contribute to transcriptional activation of an individual gene in a specific cell type, at a specific time, and for a specific length of time during the development of complex eukaryotic organisms. In the past, *Drosophila* has been used as a model system to study other aspects of transcriptional regulation. Elegant studies to understand the role of *cis*-acting elements (enhancer and promoter sequences) in transcriptional regulation have been performed in the *Drosophila* embryo, where temporally and spatially restricted patterns of transcription contribute to the establishment of a segmented body plan. In addition, similar strategies have been effectively applied to the study of gene-specific enhancer binding factors.

We plan to study the role of TAFs during transcriptional activation. TAFs are integral components of the basal transcription factor IID (TFIID) that bind to gene-specific enhancer binding factors and mediate transcriptional

activation *in vitro*. Combined *in vitro* and *in vivo* analyses of mutant TAF proteins indicate that interactions with enhancer binding proteins and assembly into the TFIID complex are also critical for their function *in vivo*. To identify mRNAs that are down-regulated in TAF mutant flies, we will use differential display in conjunction with Northern blot and *in situ* hybridization analyses. The identification of *in vivo* targets of TAFs will address questions concerning how generally TAFs function, whether different combinations of TAFs are required for transcription from different genes, and whether TAFs are required for transcription from promoters with particular *cis*-regulatory sequences, as has been suggested by studies of TAFs in yeast.

Using a technique called differential display, we propose to identify genes that are differentially expressed in flies carrying mutations in one of the two copies of either TAF60 or TAF110 (two of the eight identified *Drosophila* TAFs). There is preliminary evidence in the literature that TAFs are not general transcriptional activators but instead display gene specificity *in vivo*. Analysis of TAF60 and TAF110 mutant flies has shown that the transcription of a number of genes is down-regulated, including *sev*, which is required for eye development, and *hunchback*, which is required for embryonic development. However, the transcription of other genes is not affected, such as *Rh*3 opsin in the eye and *huckebein* at the posterior pole of the embryo. In addition, studies of temperature-sensitive hamster cell lines, in which mutations in TAF250 cause a block in cell cycle progression, reveal that there is not a global repression of transcription in the cell, but that specific genes are down-regulated at the nonpermissive temperature. These results have recently been confirmed by studies in yeast in which inactivation or conditional elimination of TAF activity also results in a cell cycle block with no apparent rapid effect on general transcription levels. The yeast results also suggest that TAFs may play more important roles for transcription from genes that contain weak TATA promoter elements.

We will isolate total mRNA from wild-type and TAF mutant flies at different stages of development and use the polymerase chain reaction (PCR) in the differential display procedure to identify mRNAs whose expression is altered in mutant flies. The differential expression of the identified genes will then be confirmed by Northern blot analysis and *in situ* hybridization to embryos. This method allows for "random" screening of gene expression levels. Another approach we will take is to look at the expression level of a set of known genes, such as those involved in cell cycle progression. These approaches will allow us to compile a list of genes that require a particular TAF to achieve the appropriate transcription level. This list will provide answers to questions such as: (1) are TAF60 and TAF110 required for the transcription of the same set of genes; (2) are there classes of genes (i.e., those involved in a specific biological process) that require a particular TAF; (3) are there *cis*-acting sequences that direct the use of a particular TAF; (4) does transcription of a specific gene require the same set of TAFs at all stages of development; and (5) are there genes whose transcription is negatively regulated by TAFs.

CELL BIOLOGY AND METABOLISM BRANCH

Chief
Richard D. Klausner, M.D.

Principal Investigators
Juan S. Bonifacino, Ph.D.
Tracey A. Rouault, M.D.
Lawrence E. Samelson, M.D.

Tenure-Track Investigators
Jennifer Lippincott-Schwartz, Ph.D.
Gisela Storz, Ph.D.
David Wassarman, Ph.D.

Senior Fellows
Jeffrey Humphrey, M.D.
Jorge Moreira, M.D.
Caroline Philpott, M.D.
Daniel Yuan, M.D.

Staff Scientist
Robert Stearman, Ph.D.

Post-Doctoral Fellows
Ruben Claudio Aguilar, Ph.D.
Charles Allerson, Ph.D.
Edward Bruggeman, Ph.D.
Geoffrey Chen, Ph.D.
Esteban Dell'Angelica, Ph.D.
Steven Drake, Ph.D.
Tiit Land, Ph.D.
Stephen Lee, Ph.D.
Pierre Ledoux, Ph.D.
Maria Merlotti, Ph.D.
Esther Meyron-Holtz, Ph.D.
Chris Mullins, Ph.D.
Aoyagi Norikazu, Ph.D.
Chean Ooi, Ph.D.
Arnim Pause, Ph.D.
John Presley, Ph.D.
Joanne Sloan-Lancaster, Ph.D.
Wing-Hang Tong, Ph.D.
Jeroen Van Leeuwen, Ph.D.
Karen Wassarman, Ph.D.
Nathan Wolins, Ph.D.
Tetsuo Yamazaki, M.D., Ph.D.

Kristien Zaal, Ph.D.
Aixia Zhang, Ph.D.
Weiguo Zhang, Ph.D.
Ming Zheng, M.D.

Pre-Doctoral Fellows
Nelson Cole
Jan Ellenberg
Marie Christine Fournier
Jason Kitchen
Heather Kontaxis
Rebecca LaFond
William Land
Jared Rashford
Leah Russin

Trevor Shar
Ronald Trible

Guest Researchers
Victor Gordeuk, M.D.
Sandra Hasstedt
Koret Hirschberg, Ph.D.
Hae-Yeong Kim, Ph.D.
Chad Miller
Sarah Nehls
Brett Peterson
Ali Salim
Jessica Shafer
Ronald L. Wange, Ph.D.
Jane Wey

ANNUAL BIBLIOGRAPHY

Addess KJ, Basilion JP, Klausner RD, Rouault TA, Pardi A. Structure and dynamics of the iron responsive element RNA: implications for binding of the RNA by iron regulatory proteins. J Mol Biol 1997;274:72-83.

Aguilar RC, Ohno H, Roche KW, Bonifacino JS. Functional domain mapping of the clathrin-associated adaptor medium chains μ1 and μ2. J Biol Chem 1997;272:2760-6.

Altuvia S, Weinstein-Fischer D, Zhang A, Postow L, Storz G. A small, stable RNA induced by oxidative stress: Role as a pleiotropic regulator and antimutator. Cell 1997;90:43-53.

Cole NB, Sciaky N, Marotta A, Song J, Lippincott-Schwartz J. Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral ER exit sites. Mol Biol Cell 1996;7:631-50.

Cole NB, Smith C, Sciaky N, Terasaki M, Edidin M, Lippincott-Schwartz J. Diffusional mobility of Golgi proteins in membranes of living cells. Science 1996;273:797-801.

Dell'Angelica EC, Ohno H, Ooi CE, Rabinovich E, Roche K, Bonifacino JS. AP-3: an adaptor-like protein complex with ubiquitous expression. EMBO J 1997;16:917-28.

Dell'Angelica EC, Ooi CE, Bonifacino JS. β3A-adaptin, a subunit of the adaptor-like complex AP-3. J Biol Chem 1997;272:15078-84.

Eischen CM, Williams BL, Zhang W, Samelson LE, Lynch DH, Abraham RT and Leibson PJ. ZAP-70 tyrosine kinase is required for the up-regulation of Fas ligand in activation-induced T cell apoptosis. J Immunol 1997;159:1135-9.

Ellenberg J, Siggia ED, Moreira JE, Smith CL, Presley JF, Worman HJ, Lippincott-Schwartz J. Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. J Cell Biol 1997;138:1193-206.

Finegold AA, Shatwell KP, Segal AW, Klausner RD, Dancis A. Intramembrane bis-heme motif for transmembrane electron transport conserved in a yeast iron reductase and the human NADPH oxidase. J Biol Chem 1996;271:31021-4.

Gangaidzo IT, Moyo VM, Khumalo H, Saungweme T, Gomo Z, Rouault TR, Gordeuk VR. Hepatitis C virus in Zimbabwe. The Central African Journal of Medicine 1997;43:122-5.

Germain RN, Bikoff EK, Bonifacino JS, Castellino F, Han R, Marks MS, Romagnoli P, Zhong G. Invariant chain regulation of MHC class II function in the secretory and endocytic pathways. In: A Svejgaard, S Buus, L Fugger, eds. Alfred Benzon Symposium, 1996;40:1-13.

Gnarra JR, Duan DR, Weng Y, Humphrey JS, Chen DYT, Lee S, Pause A, Dudley CF, Latif F, Kuzmin I, Schmidt L, Duh FM, Lerman MI, Zbar B, Klausner RD, Linehan WM. Molecular cloning of the von Hippel-Lindau tumor suppressor gene and its role in renal carcinoma. Biochim Biophys Acta, 1996;1242:201-10.

Gnarra JR, Ward JM, Porter FD, Wagner JR, Devor DE, Grinberg A, Emmert-Buck MR, Westphal H, Klausner RD, Linehan WM. Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. Proc Natl Acad Sci USA 1997;94:9102-7.

Gnarra JR, Zhou S, Merrill MJ, Wagner J, Krumm A, Papavassiliou E, Oldfield E, Klausner RD, Linehan WM. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the VHL tumor suppressor gene product. PNAS 1996;93:10589-94.

Humphrey J, Klausner RD, Linehan WM. von Hippel-Lindau syndrome: hereditary cancer arising from inherited mutations of the VHL tumor suppressor gene. Cancer Treat Res 1996;88:13-39.

Hung IH, Suzuki M, Yamaguchi Y, Yuan DS, Klausner RD, Gitlin JD. Biochemical characterization of the Wilson disease protein and functional expression in the yeast Saccharomyces cerevisiae. J Biol Chem 1997;272:21461-6.

Jamieson DJ, Storz G. Transcriptional regulators of oxidative stress responses. In: Scandalios J, ed. Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbor: CSHL Press, 1997;91-115.

Kim HY, LaVaute T, Iwai K, Klausner RD, Rouault TA. Identification of a conserved and functional iron-responsive element in the 5'UTR of mammalian mitochondrial aconitase. J Biol Chem 1996;271,24226-30.

Kirchhausen, T., Bonifacino, JS, Riezman, H. Linking cargo to vesicle formation: receptor tail interactions with coat proteins. Current Op Cell Biol 1997;9:488-95

Klausner RD, Rouault TA. The molecular basis of iron metabolism. Cancer Treat Res 1996;88:13-39.

Klomp LW, Lin SJ, Yuan DS, Klausner RD, Culotta VC, Gitlin JD. Identification and functional expression of HAHI, a novel human gene involved in copper homeostasis. J Biol Chem 1997;272:9221-6.

Linehan WM, Klausner RD. Renal carcinoma. In: CR Scriver, AL Beaudet, WS Sly, D Valle, eds. The metabolic basis of inherited disease. New York: McGraw-Hill, 1996.

Lippincott-Schwartz J. Membrane traffic and compartmentalization within the Secretory Pathway. In: Schultz et al, eds. Molecular biology of membrane transport disorders. Plenum Press, 1996;1-9.

Lippincott-Schwartz J, Smith CL. Insights into secretory and endocytic membrane traffic using green fluorescent protein chimeras. Curr Opin Neurobiol 1997;7:631-9.

Marks MS, Ohno H, Kirchhausen T, Bonifacino JS. Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores. Trends Cell Biol 1997;7:124-8.

Moyo VM, Gangaidzo IT, Gomo ZA, Khumalo H, Saungweme T, Kiire CF, Rouault, T, Gordeuk VR. Traditional beer consumption and the iron status of spouse pairs from a rural community in Zimbabwe. Blood 1997;89:2159-66.

Ohno H, Aguilar RC, Fournier MC, Henneke S, Cosson P, Bonifacino JS. Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family. Virology 1997;238:305-15.

Ooi CE, Moreira JE, Dell'Angelica EC, Poy G, Wassarman DA, Bonifacino JS. Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the *Drosophila* eye color mutant *garnet*. EMBO J 1997;16:4508-8.

Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD. Copper-dependent degradation of the *S. cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. EMBO J 1996;15:3515-23.

Ota Y, Beitz LO, Scharenberg AM, Donovan JA, Kinet J-P, and Samelson LE. Characterization of Cbl tyrosine phosphorylation and a Cbl-Syk complex in RBL-2H3 cells. J Exp Med 1996:184:1713-23.

Ota Y, Samelson LE. The protein product of the proto-oncogene c-*cbl*: a negative regulator of the Syk protein tyrosine kinase in RBL-2H3 cells. Science 1997;276:418-20.

Pause A, Aso T, Linehan WM, Conaway JW, Conaway RC, Klausner RD. Interaction of the von Hippel-Lindau tumor suppressor gene product with elongin. Methods in Enzymology, 1996;274:436-41.

Pause A, Lee S, Worrell RA, Chen DYT, Burgess WH, Linehan WM, Klausner RD. The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc Natl Acad Sci USA 1997;94:2156-61.

Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal K, Lippincott-Schwartz J. ER to Golgi transport visualized in living cells. Nature 1997;389:81-5.

Rosner JL, Storz G. Regulation of bacterial responses to oxidative stress. Curr Top Cell Regul 1997;35:163-77.

Rouault TA, Klausner RD. The impact of oxidative stress on eukaryotic iron metabolism. In: Feige et al., eds. Stress-inducible cellular responses. Birkhäuser/Springer, 1996;183-99.

Rouault TA, Klausner RD. Regulation of iron metabolism in eukaryotes. In: Stadtman et al., eds. Current topics in cellular regulation. Academic Press, 1997;Vol.35:1-19.

Rouault TA, Klausner RD. Post-transcriptional regulation of genes of iron metabolism in mammalian cells. J Bioinorganic Chem 1996;1:494-8.

Sciaky N, Presley J, Smith C, Zaal KJM, Cole N, Moreira JE, Terasaki M, Siggia E, Lippincott-Schwartz J. Golgi tubule traffic and the effects of brefeldin A visualized in living cells. J Cell Biol 1997;139:1137-56.

Shapiro J, Sciaky N, Lee J, Bosshart H, Angeletti RH, Bonifacino JS. Localization of endogenous furin in cultured cell lines. J Histochem Cytochem 1997;45:3-12.

Shiratori T, Miyatake S, Ohno H, Nakaseko C, Bonifacino JS, Saito T. Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with the clathrin-associated adaptor complex AP-2. Immunity 1997;6:583-9.

Shatwell KP, Dancis A, Cross AR, Klausner RD, Segal AW. The FRE1 ferric reductase of *Saccharomyces cerevisiae* is a cytochrome *b* similar to that of NADPH oxidase. J Biol Chem 1996;271:14240-4.

Sloan-Lancaster J, Zhang W, Presley J, Williams BL, Abraham RT, Lippincott-Schwartz J, Samelson LE. Regulation of ZAP-70 intracellular location: visualization with the green fluorescent protein. J Exp Med 1997;186:1713-24.

Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A. A permease-oxidative complex required for high-affinity iron uptake in yeast. Science 1996;271:1552-7.

Storz G, Polla BS. Transcriptional regulators of oxidative stress-inducible genes in prokaryotes and eukaryotes. In: Feige U, Morimoto RI, Yahara I, Polla BS, eds. Stress-inducible cellular responses. Basel: Birkhäuser Verlag, 1996:239-54.

Wassarman DA, Therrien M. Ras1-mediated photoreceptor development in *Drosophila*. Adv Dev Biochem 1997;5:1-41.

Watts JD, Brabb T, Bures EJ, Wange RL, Samelson LE, and Aebersold R. Identification and characterization of a substrate specific for the T cell protein tyrosine kinase ZAP-70. FEBS Letters 1996;398:217-22.

Yamaguchi-Iwai Y, Serpe M, Haile D, Yang W, Kosman DJ, Klausner RD, Dancis A. Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. J Biol Chem 1997;272:17711-8.

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner RD. Iron-regulated DNA binding by the AFT1 protein controls the iron regulation in yeast. EMBO J 1996;15:3377-84.

Yang M, Ellenberg J, Bonifacino JS, Weissman AM. The transmembrane domain of a carboxyl-terminal anchored protein determines localization to the endoplasmic reticulum. J Biol Chem 1997;272:1970-5.

Yuan DS, Dancis A, Klausner RD. Restriction of copper export in *Saccharomyces cerevisiae* to a late-Golgi or post-Golgi compartment in the secretory pathway. J Biol Chem 1997;272:25787-93.

DEVELOPMENTAL ENDOCRINOLOGY BRANCH

Carolyn A. Bondy, M.D., Chief

The DEB investigates fundamental issues in human reproduction, growth, and development. Researchers within this branch employ genetic, biochemical and physiological analyses to elucidate the molecular mechanisms underlying the wide array of clinical disorders available for study in our reproductive, pediatric and adult endocrinology clinics. Current projects focus on the role of sex steroids and growth factors in regulating growth and reproduction, on molecular mechanisms of glucocorticoid resistance, on genetic and epigenetic interactions in the development of obesity and diabetes mellitus, on basic mechanisms of ovarian follicular function and dysfunction in disorders such as premature menopause and polycystic ovary syndrome, on the genetic origins of endocrine tumors, on the roles of CRH and vasopressin in stress and immune responses, and on improving the diagnosis and treatment of congenital adrenal hyperplasia, hypoparathyroidism, Cushing's syndrome, and menopause.

MECHANISMS OF GROWTH FACTOR ACTION

The Section on Growth and Metabolism, led by ***Carolyn Bondy***, investigates fundamental cellular and physiological processes regulating mammalian growth and reproduction. To further this aim, members of this Section have focused on the biological roles and mechanisms of action of insulin-like growth factor-I (IGF-I). Their studies have provided an essential road map of IGF functions at the local level and shaped a new view of the IGF system as a local regulator of cellular metabolic and anabolic activity. The ultimate goal of this research is to apply knowledge of IGF's biological roles to the diagnosis and treatment of disorders of human growth and neoplasia.

Brain Development. This group had previously shown that IGF-I and its cognate receptor are highly expressed in the developing brain in a spatiotemporal pattern that implicates this growth-promoting peptide in dendritogenesis, synaptogenesis, and/or myelination. The recent demonstration that humans nullizygous for IGF-I are mentally retarded supports the view that IGF-I has an important role in brain development. Other investigators built a case, based on *in vitro* data, that IGF-I plays a major role in brain myelination. To test this hypothesis, Bondy and colleagues compared myelination of brains from IGF-I-targeted gene deletion ("knockout", KO) mice with that of brains from wild-type (WT) litter mates. They have now demonstrated that the myelin content and the level of expression for all the major myelin proteins is equal in WT and KO brains. Regional analysis has demonstrated that the anatomical patterns and abundance of myelin-specific protein, lipid, and gene expression are equivalent in WT and KO brains, except for myelin tracts emanating from the olfactory bulb, which are selectively attenuated in the KO mice. Further neuroanatomical analysis revealed that the olfactory bulb itself is severely underdeveloped in the IGF-I KO brain. Additional studies showed that the number, distribution and morphology of oligodendrocytes is no different in WT and KO brains. Hence, it appears that IGF-I does not play a major role in brain myelination. The reduction in olfactory system myelination is due to hypoplasia of the olfactory bulb in the IGF-I KO mouse rather than to a primary defect in myelination.

Further studies conducted in this lab have established that endogenous brain IGF-I plays an important role in the regulation of neuronal metabolism. The highly metabolically active brain relies almost exclusively on glucose to supply fuel. Despite the fact that the brain constitutes only about 2% of body weight, it accounts for over 20% of the body's glucose consumption. The manner in which brain glucose metabolism is regulated, however, is poorly understood. Little if any insulin is synthesized within the brain and very little crosses the blood brain barrier. Based on Bondy's demonstration that neuroanatomical patterns of IGF-I and IGF-I receptor expression track regional brain glucose utilization, it was hypothesized that locally produced IGF-I serves to regulate neuronal glucose metabolism.

Using the *in vivo* radiolabelled 2-deoxyglucose uptake methodology, it has been demonstrated that there is a highly significant reduction in glucose utilization during postnatal brain development in the IGF-I KO mouse. This reduction is most pronounced in those brain areas in which IGF-I is normally expressed. *In vitro* studies

using fresh synaptosomes prepared from IGF-I KO and WT litter mates showed a 50% decrease in glucose uptake in the KO, which can be corrected by incubation with IGF-I.

Uterine and Mammary Growth. IGF-I, which has been characterized as a "G1-progression factor" for fibroblast cell lines, has also been implicated as a local mediator of estradiol's (E2) mitogenic effects upon the uterus. To test the hypothesis that IGF-I is required for E2-induced uterine cell proliferation and to identify the cell cycle states which are sensitive to IGF-I *in vivo*, Bondy and co-workers compared E2's effects on the uteri of IGF-I KO and WT litter mate mice. E2 treatment induces robust and equal increases in the proportion of cells in S-phase, but the mitotic index is much lower in KO uteri than in WT uteri. Thus, it appears that E2-induced entry into the mitotic cycle and progression through G1-phase to S-phase proceed normally, but that progression from S-phase to mitosis is significantly impaired in the absence of IGF-I. These data show that IGF-I is an important mediator of E2's mitogenic effects on the uterus with a critical role, not in G1 progression, but in completing the cell cycle from S-phase to M-phase.


Carolyn Bondy

The decline of growth hormone (GH) and IGF-I production during aging has been likened to the menopausal decrease in gonadal steroids, and GH treatment of aging individuals has been recommended to restore the lean tissue anabolism characteristic of youth. In addition to anabolic effects on musculoskeletal tissues, however, GH also stimulates mammary glandular growth in some species, although its effects on primate mammary growth are unclear. Some clinical observations implicate GH in human mammary growth, e.g., gynecomastia, that occurs in some patients treated with GH, and acromegaly is associated with an increased incidence of breast cancer. To investigate the effects of GH treatment on mammary tissue in a model relevant to aging humans, the group treated aged female rhesus monkeys with GH or saline diluent for seven weeks. We found a highly significant increase in mammary glandular tissue abundance and epithelial cell proliferation in all the GH-treated groups. These mitogenic effects correlate directly with both circulating GH and IGF-I levels, suggesting that either GH or its downstream effector IGF-I stimulates primate mammary epithelial proliferation.

To determine if GH or IGF-I could be acting directly on mammary epithelium, the investigators analyzed the cellular localization of GH-, prolactin- and IGF-I-receptor mRNAs in monkey mammary gland. GH receptor mRNA was found to be expressed in mammary adipose but not in stromal or epithelial cells, while prolactin receptor (PRLR, which binds to GH as avidly as does the GHR) mRNA is abundant in mammary epithelium. IGF-I receptor mRNA also concentrates in mammary epithelium. Thus, GH treatment results in significant mammary hyperplasia in aging non-human primates, either due to a direct action of GH on mammary epithelium through the PRLR or due to an indirect effect mediated by IGF-I through its cognate receptor in mammary epithelium. These findings suggest that GH supplementation may stimulate unwanted mammary epithelial hyperplasia in aging individuals.

MOLECULAR BIOLOGY OF DIABETES MELLITUS

The Unit on Genetics and Hormone Action, led by ***Domenico Accili***, studies the genetic basis of non-insulin-dependent diabetes mellitus (NIDDM). NIDDM is currently viewed as a complex metabolic disorder with multiple causes. The metabolic hallmarks of NIDDM are peripheral insulin resistance and impaired insulin secretion. The two defects are intimately intertwined in most NIDDM patients. However, in longitudinal studies of patients at high risk of developing NIDDM, it appears that insulin resistance is the first identifiable metabolic abnormality. Insulin resistance is a genetically complex trait, i.e., it is not inherited in a Mendelian fashion and simple genetic models, such as studies of candidate genes, have failed to identify the gene(s) responsible for insulin resistance. Several aspects of insulin action appear to be impaired in NIDDM, but the nature of the fundamental defect remains unclear. It is widely held that a defect at an early post-receptor step in insulin action could provide a unifying mechanism for insulin resistance. In recent years, the pace of discovery of new molecules and novel signaling mechanisms downstream of the insulin receptor (IR) has quickened considerably.

However, studies of genes encoding proteins that participate in the insulin signaling systems have failed to implicate one or more of these genes as a major candidate for the etiology of NIDDM. It is possible that, because of genetic heterogeneity, different genes are responsible for insulin resistance in different patients. Furthermore, the functional impairment caused by some genetic variations might be too subtle to be detected by the assays at our disposal, or may require the combined interactions of other mutant genes to give rise to the diabetic phenotype. To clarify these issues, Accili and coworkers are trying to dissect the genetic components of insulin resistance, using mouse and human genetic models.

Targeted Mouse Mutants with Insulin Resistance and Diabetes. This project focuses on the characterization of mice with targeted mutations in the pathway of insulin action. To generate a mouse model of insulin resistance, scientists in the Unit initially introduced a mutant allele of the insulin receptor into mice. Mice born without functional insulin receptors have normal features at birth; their intrauterine growth and development appear to be normal. However, upon feeding they develop dramatic metabolic alterations accompanied by hyperglycemia and hyperketonemia. Within a few days, mutant mice die of diabetic ketoacidosis (DKA). Thus, lack of insulin receptors in mice impairs fuel metabolism. The early post-natal death caused by inactivation of the insulin receptor gene is reminiscent of other targeted inactivations of genes important for fuel metabolism, such as glucokinase and C/EBP-a.

Polygenic Mouse Model of Non-Insulin-Dependent Diabetes Mellitus. An important contribution of the Unit has been the development, in collaboration with C.R. Kahn's laboratory at the Joslin Diabetes Center, of a polygenic mouse model of NIDDM. In these experiments, mice heterozygous for an insulin receptor null allele have been crossed with mice heterozygous for a null mutation of the gene encoding IRS-1, a major substrate of the insulin receptor kinase. IRS-1 is thought to mediate insulin and IGF-1 signaling by way of interactions with adaptor molecules such as phosphatidylinositol (PI)-3-kinase, grb-2, sos, syp (tyrosine phosphatase 1B), and many others. Mice lacking IRS-1 are growth retarded and mildly insulin resistant, consistent with a model in which IRS-1 mediates both the actions of insulin and IGF-1 receptors. Mice heterozygous for both an insulin receptor and an IRS-1 mutant allele develop all the hallmarks of insulin resistance: hyperinsulinemia and hyperplasia of pancreatic beta cells. Within two months of birth, they become frankly hyperglycemic, thus resembling very closely the pathogenesis of human diabetes. These findings are consistent with a polygenic mode of inheritance of NIDDM. This mouse model of NIDDM represents a significant advance over existing animal models of this condition, such as the ob/ob and db/db mice, which are primarily mouse models of obesity. In a broader context, lessons learned from this mouse model are likely to have a substantial impact on current gene mapping strategies in common diseases, because they demonstrate how two genes can interact epistatically in the pathogenesis of these disorders.


Domenico Accili

Insulin Receptors in Embryonic Development. The role of insulin in fetal life is a matter of debate. In addition to the growth retardation observed in cases of extreme insulin resistance, macrosomia is a well recognized complication of fetal hyperinsulinemia, a metabolic consequence of the diabetic pregnancy. The relatively normal phenotype of nullizygous insulin receptor mice, however, suggests that during gestation, insulin receptors can be replaced by other receptors. Because of the close structural and functional homology between insulin and IGF-1 receptors, the IGF-1 receptor is a prime candidate to compensate for functions normally exerted by insulin receptors. This group has collaborated with investigators at Columbia University to test the hypothesis that the insulin receptor is the receptor responsible for survival of mice lacking both the IGF-1 and IGF-2 receptors, which are smaller than mice lacking either receptor alone. Mice lacking all three receptors (insulin, IGF-1 and IGF-2) are even smaller than the double insulin/IGF-1 knock-out mice. These data indicate that insulin receptors play an ancillary role in embryonic growth. It is likely that these actions of the insulin receptor are mediated in response to IGF-2 binding, since the knock-outs of the two ligands, insulin and IGF-1, suggest that the actions of these two hormones are only mediated by their respective receptors. It remains to be determined whether the growth-promoting actions of the insulin receptor are mediated by holodimeric insulin receptors (composed of two a-subunits and two b-subunits) or by heterodimeric receptors composed of

an insulin receptor a/ b monomer and an IGF-1 receptor a/ b monomer. Experiments to address this question are under way.

BONE GROWTH AND MINERAL METABOLISM

The Unit on Growth and Development, led by ***Jeffrey Baron***, investigates the cellular and molecular mechanisms governing bone growth and mineral metabolism. This knowledge is applied to improve medical treatment in disorders of bone growth and in disorders of mineral metabolism. Through the study of longitudinal bone growth, the investigators also seek to uncover general principles of developmental biology, since the cellular processes underlying bone growth, such as cell proliferation, terminal differentiation, angiogenesis, and cell migration, are essential for development in other tissues.

Ca^{2+}-Sensing Receptor Mutations. This research group identified Ca^{2+}-sensing receptor (CaR) gene mutations in families with autosomal dominant hypoparathyroidism. All were heterozygous missense mutations. The resulting phenotype varies from asymptomatic to life-threatening, depending on the specific mutation. The investigators also demonstrated that sporadic hypoparathyroidism can be caused by *de novo* CaR mutations. Identification of these mutations in sporadic cases defines the risk to the patient's future offspring, thus allowing appropriate genetic counseling.

Because the CaR inhibits parathyroid hormone secretion, it was predicted that these mutations activate the CaR. To confirm this prediction and to explore the mechanism of activation, the mutations were introduced into the human CaR cDNA and expressed in cultured cells. Some of the mutations cause a left-shift in the concentration-response curve, indicating an increase in sensitivity to calcium and suggesting an increase in affinity. Three of these mutations also show an increase in maximal signal transduction capacity. This dual effect helps to define the molecular mechanisms by which G-protein coupled receptors transduce signals across the cell membrane.


Jeffrey Baron

Longitudinal Bone Growth. Fibroblast growth factors (FGF's) and FGF receptors are expressed by growth plate chondrocytes. *In vivo*, the FGF system appears to inhibit longitudinal bone growth. To explore the underlying mechanisms, this group developed an organ culture system, involving fetal rat metatarsal bone rudiments, and demonstrated that FGF-2 reduces growth plate chondrocyte proliferation, hypertrophy, and cartilage matrix production. The investigators have also demonstrated that an *in vivo* infusion of FGF-2 into the growth plate accelerates local vascular invasion and ossification of growth plate cartilage. Taken together, these data suggest that fibroblast growth factors may regulate endochondral bone formation in the growth plate at multiple levels, including chondrocyte proliferation and differentiation, angiogenesis, and ossification. These findings provide insight not only into the physiological role of FGF's in the growth plate, but also into the pathophysiology of the skeletal dysplasias caused by FGF receptor mutations.

Other current studies explore the role of retinoids in growth plate function and the mechanisms determining spatial polarity in the growth plate. Trials using growth hormone or insulin-like growth factor-1 for the treatment of idiopathic extreme short stature are also under way.

TREATMENT OF CONGENITAL ADRENAL HYPERPLASIA, SHORT STATURE, HYPOPARATHYROIDISM

New Approaches to the Treatment of Short Stature. The Developmental Endocrinology Branch investigates the basic mechanisms and clinical disorders of growth and puberty. Four major clinical trials are in progress that are designed to evaluate new approaches to the treatment of short stature. The first tests the hypothesis that prolonging the growth period by delaying puberty can enhance adult height. An interim analysis indicates that delay of puberty by an average of 3.6 years increases adult height by 6.8 cm compared with the randomized, placebo-treated control. The second clinical trial tests the hypothesis that supplemental growth hormone (GH),

with or without low-dose ethinyl estradiol, will increase the adult height of girls with Turner syndrome. Since girls with Turner syndrome average more than 3 SD below the normal mean height, short stature is the major concern of patients with this disorder. The outcome variables include data on both safety and efficacy, and will allow an assessment of the risk-to-benefit ratio of this new treatment. The third clinical trial tests the hypothesis that supplemental GH will increase the adult height of children with non-GH-deficient short stature (constitutional, familial, or idiopathic short stature). The fourth clinical trial tests the hypothesis that recombinant human insulin-like growth factor-1 can increase the growth velocity of children with Laron short stature and with idiopathic short stature. The number of short children in the United States who are potential candidates for treatment is about 1,000,000, which underscores the need for a rigorous assessment of risk-to-benefit. Thus, the answers sought by these trials have important clinical, public health, and scientific significance.

Clinical Studies in Turner Syndrome. Studies of a large cohort of girls with Turner syndrome have yielded new behavioral findings during the previous year. Analysis of behavior and social relationships in Turner girls revealed weaker social relationships, school performance, and self-esteem compared with control girls matched for age, socioeconomic status, and verbal IQ. With administration of estrogen beginning at age 12, however, we observed improved self-concept, both by self and parental report, so that by age 14-15 the girls with Turner syndrome resembled the normal controls. This improvement was not seen in a comparison group of Turner girls in whom estrogen treatment was delayed. These findings underscore the need to initiate estrogen replacement therapy by age 12-14 years in this population. Second, cognitive studies of the Turner subjects has shown a characteristic profile of impaired performance on tests of spatial recall, recognition, and reasoning despite normal verbal ability and performance. This cognitive profile is consistent from childhood through adolescence. The magnitude of the impairment is about one standard deviation unit, which implies that about 16% of Turner girls fall below the lower 95% confidence limit for normal girls. These cognitive findings suggest that spatial abilities depend on X-chromosome genes. Ongoing studies are attempting to localize these genes by determining the relationship between genotype and cognitive phenotype in Turner girls with unusual karyotypes involving partial deletion of one X-chromosome. From a social and educational standpoint, these studies suggest subtle differences in the social and cognitive performance of girls with Turner syndrome that may respond to educational approaches that are more closely tailored to their particular needs.

Treatment of Familial Male Precocious Puberty. A new approach to the treatment of FMPP has proven effective in normalizing the rate of growth and bone maturation and in preventing acne, spontaneous erections, and aggressive behavior. The treatment combines an antiandrogen (spironolactone) with an inhibitor of androgen-to-estrogen conversion (testolactone) to block estrogen effects on epiphyseal growth. At present, the long-term effects of this new treatment are being evaluated, and attempts are being made to improve treatment with more potent aromatase inhibitors, and to treat secondary central precocious puberty, when it complicates FMPP, with an LHRH analog as well.

Treatment of Congenital Adrenal Hyperplasia. Accelerated growth in FMPP is caused by elevated secretion of androgen and estrogen, which is the same mechanism that causes accelerated growth and early epiphyseal fusion in congenital adrenal hyperplasia (CAH). Thus, workers in the DEB have begun a new trial to test whether the combination of an antiandrogen (flutamide) and an inhibitor of androgen-to-estrogen conversion (testolactone) would be effective in CAH. The short-term results of this regimen have shown improved control of growth rate, weight gain, and bone maturation, and reduced risk of glucocorticoid complications because the hydrocortisone dose is lower. A longer-term study of this new regimen is ongoing. Laboratory studies in mice with CAH due to 21- hydroxylase deficiency are testing the hypothesis of an increased number of hypothalamic CRH neurons due to decreased glucorticoid negative feedback *in utero*. This hypothesis would help explain the clinical observation of apparent resistance to hypothalamic suppression by glucocorticoid in CAH. A second initiative attempts to correct CAH in 21-hydroxylase-deficient mice through gene therapy.

New Treatment for Hypoparathyroidism. Hypoparathyroidism is one of the few remaining hormonal deficiencies for which hormone replacement therapy is not available. Moreover, the existing treatment, vitamin D or its analogs and calcium, lacks the full renal calcium-retaining action of parathyroid hormone (PTH), and thus treated patients often exhibit hypercalciuria. To test the hypothesis that treatment with PTH 1-34 can achieve simultaneous normalization of both serum and urine calcium, this group has conducted a randomized crossover trial of PTH 1-34 compared with calcitriol in patients with chronic hypoparathyroidism. The study showed that once-daily treatment with PTH 1-34 maintains both serum and urine calcium within the normal range. Biochemical markers of bone turnover increase significantly during PTH 1-34 treatment. Although rare, hypoparathyroidism represents a unique opportunity for the scientific study of the effect of hCG on phosphate

(IP) levels. Future studies will attempt to refine the model for mutant receptors to determine the precise structural correlates of receptor activation.

WOMEN'S HEALTH

Ovarian Function. Members of the Unit on Gynecologic Endocrinology have prospectively studied a large series of patients with premature ovarian failure and has characterized this group using state of the art imaging, and immunological and molecular analyses. These investigators have found that a minority of these women have ovarian failure of immune origin, and that in almost half the affected women ovarian follicular development could be detected by ultrasound and by rising serum estradiol levels. Biopsy of these follicles shows a consistent pattern of premature luteinization, suggesting the presence of a functional and potentially reversible disorder of folliculogenesis. Current studies are aimed at elucidating the functional deficit, with a view to designing novel therapeutic approaches to rescue or revive ovarian follicular function for these young women. In addition, recent studies on these same patients have shown that despite usual "hormone replacement therapy" typically prescribed for older women who have undergone natural menopause, two thirds of the younger women have significantly reduced bone mineral density, putting them at increased risk for fracture. It is not clear whether the osteoporosis is secondary to inadequate estrogen replacement, or, alternatively, to the fact that these women also have significantly reduced androgen levels, as the group has recently demonstrated. To investigate this issue and to optimize the reconstitution of the normal sex steroid milieu for young women with premature menopause, a prospective trial comparing estradiol and estradiol plus testosterone replacement therapy is being launched.

Menopause. Bondy's group has developed a non-human primate model to elucidate tissue-specific effects of the major menopausal hormone replacement therapy regimens. Ovariectomized rhesus monkeys are treated with subcutaneous implants containing estradiol (E2) alone, E2 plus progesterone (P4), E2 plus testosterone, tamoxifen or saline. Uterine, vascular, mammary, and other hormone-sensitive tissues are evaluated for changes in expression of growth factors and their receptors, as well as for markers of cellular proliferation and differentiation. In the first data from this study, the effects of sex steroids on the IGF system in the primate myometrium were analyzed. IGF-I and IGF-I receptor mRNAs are co-expressed by smooth muscle cells, suggesting that IGF has autocrine/paracrine effects in stimulating myometrial growth. IGF-I mRNA is barely detectable in control myometrium, is significantly increased by E2 treatment and is augmented even more by combination E2 and P4 treatment. The Ki-67 proliferation-specific antigen-positive myometrial nuclei are also significantly increased by E2 and are augmented even more by E2 plus P4 treatment, with a highly significant positive correlation between local IGF-I mRNA concentration and local Ki-67 positive cell count. These data implicate local IGF-I action in both estrogen-induced and progesterone-induced myometrial growth.

Lynnette Nieman

Obesity. Obesity affects approximately one third of the female population in the United States and is even more prevalent among African-American women. Since African-American women are at substantially greater risk for developing complications of obesity including hypertension, diabetes mellitus, nephropathy, retinopathy, vascular disease, and death, compared with Caucasian women, the elucidation of differences in pathophysiological mechanisms related to obesity in these populations is of great medical consequence. Substantial evidence suggests that risk factors and therapeutic approaches derived from the study of Caucasians may not be accurate or effective when applied to individuals of differing race. Our clinical studies have demonstrated that significant differences are found in anatomical patterns of fat deposition, insulin sensitivity, and basal metabolic rate in African-American and Caucasian women and girls. For example, it was found that visceral adiposity is relatively lower in African-American females and that insulin resistance is correlated to the amount of subcutaneous but not visceral adipose tissue. These findings indicate that the risk factors identified in studies of predominantly Caucasian populations may not be relevant for other genetic groups, and may not, in fact, represent true pathogenetic factors. Current studies are directed at evaluating the

genetic, physiological, metabolic, and behavioral factors involved in the ontogeny of obesity in children of both races.

Endometrial Physiology. The Unit on Reproductive Medicine, led by ***Lynette Nieman***, has focused in recent years on investigating factors regulating endometrial receptivity. Progesterone is essential for the development of a functional secretory endometrium. Abnormal endometrial development, whether because of inadequate progesterone secretion or because of inadequate endometrial responsiveness, may underlie infertility and multiple abortion syndromes. Recent studies have been directed at characterizing growth factors responsible for the paracrine coordination of stromal and epithelial growth. Preliminary data suggest that hepatocyte growth factor and its receptor, MET, are present in the endometrium of normally cycling women, suggesting that this growth factor may play a role in epithelial differentiation. Clinical studies employ pharmacological anti-progesterone agents to elucidate cellular and biochemical mechanisms of progesterone action on the luteal phase endometrium.

CUSHING'S SYNDROME

The Developmental Endocrinology Branch has made major contributions to the differential diagnosis of hypercortisolism. The CRH test and inferior petrosal sinus sampling (IPSS) have been established as major diagnostic tools in the identification of pituitary adenomas causing Cushing's disease. In recent studies, Lynnette Nieman and colleagues have evaluated the utility of plasma cortisol measurements to discriminate between pseudo-Cushing's syndrome and Cushing's syndrome in 260 patients. A single cortisol measurement at midnight correctly distinguished 96% of patients with Cushing's syndrome from normal individuals or those with pseudo-Cushing's syndrome.

Nieman and co-workers are now evaluating the effectiveness of internal jugular vein (IJV) sampling as a substitute for the more invasive and technically demanding inferior petrosal sinus (IPS) sampling to distinguish patients with Cushing's disease from those with ectopic adrenocorticotrophic hormone (ACTH) syndrome. Preliminary data in 20 patients with surgically proven Cushing's disease showed a sensitivity of 90% for IJV sampling, and 95% for IPS sampling. Further validation of this diagnostic test would allow local medical centers without expertise in IPSS to directly localize the source of ACTH hypersecretion in ACTH-dependent hypercortisolism, whereas at present IPSS is offered in only a few highly specialized centers.

HEREDITARY ENDOCRINE DISORDERS

The Section on Pediatric Endocrinology, headed by ***George Chrousos***, also seeks to determine the molecular basis for genetic disorders of the HPA axis and of the reproductive system. Thus, the molecular mechanisms of the hereditary diseases congenital isolated glucocorticoid deficiency, glucocorticoid resistance, testicular and ovarian LH resistance, and aromatase excess were elucidated by identifying pathogenetic defects of the ACTH, glucocorticoid, LH receptor, and aromatase genes, respectively.

Molecular Analysis of the ACTH Resistance Syndromes. ACTH regulates glucocorticoid secretion by the adrenal cortex via its recently cloned receptor. This membrane protein belongs to the superfamily of G protein-coupled receptors, forming its own distinct class with the receptors for αMSH. This group mapped the gene of the ACTH receptor in the short arm of chromosome 18, and studied families with hereditary isolated glucocorticoid deficiency (congenital insensitivity to ACTH) for potential defects of this gene. We found that point mutations resulting in premature termination of the receptor or nonconservative amino acid substitutions in key functional areas of the molecule cause the disease. Heterozygote carriers were found to be clinically healthy but can be revealed by CRH testing, since they display markedly exaggerated responses of ACTH to this hormone. In a similar syndrome, which in addition to ACTH resistance, includes alacrima and achalasia (Triple A Syndrome), we found an intact ACTH receptor gene. However, obligate heterozygotes for this syndrome also have abnormal responses to CRH stimulation.

Description and Molecular Analysis of the Aromatase Excess Syndrome. Normally, estrogen is produced from androgen precursors with the help of an enzyme, aromatase. This group recently described the third family in the world with the "aromatase excess syndrome." Three generations of family members were affected, and transmission is autosomal dominant. A boy with prepubertal gynecomastia and a girl with isosexual precocious puberty were the index cases in this family. Their father had also gynecomastia and short stature, while their

maternal grandmother and aunt had massive macromastia that necessitated reduction mammoplasty. The affected children responded well to treatment with an aromatase inhibitor, while the molecular defect was found to be the ectopic expression of an aberrant novel promoter, which was cloned and sequenced.

A Natural Determinant of Glucocorticoid Sensitivity in Human Tissues and Its Role in Human Pathophysiology. The glucocorticoid receptor gene, located in the long arm of chromosome 5, encodes two protein products by alternative splicing: (1) the classic glucocorticoid receptor, designated GRα; and (2) a nonligand binding variant, called GRβ. This group demonstrated that both isoforms are expressed in human tissues and that the nonligand binding receptor is present at high concentrations in normal human tissues and exerts dominant negative effects on the classic receptor. Since alteration in the glucocorticoid receptor gene splicing would produce different ratios of GRα to GRβ, one can envisage tissues changing their sensitivity to glucocorticoids via this mechanism. This might have major implications for human pathophysiology, since both glucocorticoid resistance and glucocorticoid hypersensitivity could produce disease states. The latter could be involved in metabolic disorders, such as visceral obesity or syndrome X, affective disorders, such as depression, and immune disorders characterized by suppression of the inflammatory/immune response and vulnerability to infectious diseases and/or tumors. This group showed that glucocorticoid-resistant asthma type II is associated with a major switch in the GRα to GRβ ratio towards GRβ predominance, explaining the profound resistance of these patients' cells to glucocorticoids. A similar switch was found in cells from glucocorticoid-resistant New World monkeys. Recently, this group demonstrated that one of the proteins of the HIV-1, called Vpr, acts as a co-regulator of the glucocorticoid receptor, altering the sensitivity of human cells to glucocorticoids such that viral replication is favored and acquired peripheral glucocorticoid resistance develops, a phenomenon observed in AIDS patients.

Chromosomal Mapping of the Carney Complex Loci. This group studied the genetics of Carney complex, a multiple neoplasia and lentiginosis syndrome involving the adrenal glands that is transmitted in an autosomal dominant fashion. We mapped a defective gene to within a one cMorgan region of the short arm of chromosome 2 in band p16. We also performed cytogenetic studies on the tumors of Carney patients, and demonstrated that the pathogenic gene is most likely to be an oncogene rather than a tumor suppressor gene. At this time, we are examining candidate genes in the area and identifying the appropriate yeast artificial chromosomes that would lead to isolation of the disease-causing gene.

George Chrousos

Role of CRH in Psychiatric and Developmental Disorders. Chrousos and co-workers have used CRH productively to probe central mechanisms in patients with atypical, seasonal depression, the chronic fatigue/fibromyalgia syndromes and the postpartum blues/depression syndromes. These patients have responses to CRH indicative of mild, sustained hypocortisolism of central nervous system etiology, suggesting that a spectrum of diseases exist, in which the pathophysiology is characterized by hypofunction of the central stress system. These syndromes contrast with melancholic depression, panic anxiety and anorexia nervosa, in which hyperactivity of the CRH system were earlier demonstrated by this group. The similarities between patients with Cushing's syndrome and atypical depression, also shown by this group, probably stem from a similar pathologic decrease in CRH secretion. Recently, these workers showed that preadolescent and adolescent girls with a history of sexual abuse have an HPA axis response to CRH similar to that of patients with melancholic depression or recovering anorexics. These findings suggest that major stress in children may have long-lasting effects on the HPA axis accompanied by effects on the psyche. Indeed, dysthymia or depression was found in all of the sexually abused children studied. In a social nonhuman primate model, this group demonstrated effects of parental abuse not only on the HPA axis but also on growth. This group recently demonstrated that antalarmin, a nonpeptide CRH antagonist, which crosses the blood barrier, inhibits the stress-mimicking properties of CRH, including its effects on HPA axis and behavior. This drug thus constitutes a new class of pharmacologic agents for the potential treatment of diseases characterized by CRH hypersecretion, such as melancholic depression and anorexia nervosa.

Tissue CRH. The group has demonstrated the presence of immunoreactive CRH in inflammatory tissues such as Hashimoto's thyroid, uveitis, arthritis, and ulcerative colitis and has shown that this peptide exerts proinflammatory effects. We are now investigating its regulation and relative importance in inflammation, employing the nonpeptide, CRH receptor antagonist, antalarmin. Initial studies have shown that this antagonist is specific for the type I CRH receptor and that it blocks inflammation. We had previously reported the detection of immunoreactive CRH and CRH binding sites in the ovary, and have recently demonstrated the presence of immunoreactive CRH in glandular cells of the human endometrium throughout the menstrual cycle and in stromal cells and local macrophages during the luteal phase. Functional studies are in progress to elucidate the potential function of endometrial CRH in rat implantation.

Interactions Between the Immune System and the HPA Axis. Several cytokines, including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), as well as several lipid mediators of inflammation, including platelet-activating factor and several eicosanoids, cause profound stimulation of the HPA axis, primarily by activating the CRH neuron. We recently studied IL-6 in humans. We found that this cytokine is a highly potent activator of the HPA axis and that, at high doses, it causes vasopressin secretion. At all doses it is quite benign, unlike TNF-α and IL-1. In recent studies, we demonstrated that IL-6 is a potent stimulator of ACTH release in normal subjects; we are now testing the hypothesis that administration of IL-6 may serve as a novel provocative test of the CRH neuron, allowing the differentiation of patients with true Cushing's disease, who are expected to demonstrate suppressed CRH neuron responses, from those with pseudo-Cushing's states, who are predicted to have normal CRH responsiveness. Preliminary information suggests that IL-6 may indeed be able to distinguish patients with Cushing syndrome from patients with pseudo-Cushing states (psychiatric hypercortisolism) and patients with melancholic depression from patients with atypical depression. We have also shown that glucocorticoids regulate IL-6 levels in human subjects, and that IL-6 levels are reduced in patients with Cushing's syndrome, but return to normal after the surgical cure of hypercortisolism. In fact, we have suggested that a rebound elevation in circulating IL-6 levels immediately after surgical cure of hypercortisolism may be the cause of the systemic symptoms typical of the glucocorticoid withdrawal syndrome. Thus, IL-6 participates in an immune-endocrine feedback loop, involving CRH, ACTH, and cortisol.

NEUROENDOCRINOLOGY OF STRESS

The Section of Endocrine Physiology, directed by ***Greti Aguilera***, has two major research interests: (1) the neuroendocrine mechanisms of the stress response with emphasis on the regulation of the hypothalamic pituitary adrenal (HPA) axis; and (2) the role of the renin-angiotensin system in the regulation of mineralocorticoid secretion and the effects of angiotensin II (Ang II) in other endocrine and non-endocrine systems.

Greti Aguilera

Physiological Effects of Angiotensin II. This group pioneered studies on the role of type 2 Ang II receptors during fetal development. Research by this group showed that the temporal pattern of expression of these receptors during postnatal development follows closely the differentiation of neural pathways in the brain, and nephron differentiation in the kidney, suggesting a role for Ang II in development. In the field of mineralocorticoid secretion, two important recent findings have been the demonstration of developmental changes in the regulation of aldosterone secretion, and of marked inhibitory effects of chronic stress on the expression of the rate-limiting enzyme in aldosterone biosynthesis, aldosterone synthetase. Current research focuses on elucidating the physiological role of central Ang II receptors. Studies during the past year showed that blockade of Ang receptors type-1 (AT) by icv injection of a specific antagonist, Losartan, markedly reduces the increases in plasma catecholamines in response to stress, while having no effect on the HPA axis responses.

Regulation of Pituitary ACTH Secretion by VP and CRH. This laboratory has demonstrated that vasopressin plays a predominant role in the regulation of pituitary ACTH secretion during chronic stress. This is reflected in increased VP:CRH ratios in the hypothalamus, and by a good correlation between pituitary ACTH

responsiveness and the content of vasopressin receptors in the pituitary. During the last year, major progress was achieved in our understanding of the regulation of pituitary CRH and VP receptors, which are critical for the responsiveness of the corticotroph during stress. These studies have shown that chronic stress induces increases in pituitary CRH receptor mRNA, despite sustained decreases in CRH binding. The mechanisms of these changes involve the interaction of hypothalamic CRH and VP and circulating glucocorticoids in the pituitary corticotroph. While acute injection of CRH or glucocorticoids in rats decreases CRH receptor mRNA, their simultaneous administration markedly reduces this inhibition. This indicates that interaction between CRH and low glucocorticoid levels counteracts individual inhibitory effects of these regulators alone. Such an effect probably accounts for the stimulatory effect of stress on pituitary CRH receptor mRNA.

Studies of this laboratory have shown that VP contributes to the increased responsiveness of the pituitary corticotroph during stress, and that the stimulatory effect of VP on ACTH release is insensitive to the inhibitory effect of glucocorticoids. Studies conducted during the past year have shown that long term glucocorticoid administration potentiates VP-stimulated inositol phosphate formation, probably through an increase in the coupling protein, Gq. Such an increase in receptor coupling may explain the refractoriness of VP-stimulated ACTH release to inhibition by glucocorticoids, and provides a mechanism by which VP can facilitate corticotroph responsiveness in spite of the elevated levels of plasma glucocorticoids during stress.

Glucocorticoid Receptors and the HPA Axis. An additional finding using a partial glucocorticoid receptor knock-out mouse as a model was that glucocorticoids also have facilitating effects on CRH expression at the hypothalamic level. In contrast to the stimulation of hypothalamic-pituitary corticotroph function expected in this model, CRH expression in the PVN was found to be reduced and POMC expression in the pituitary to be normal under basal or stimulated conditions. These findings suggest that low expression levels of glucocorticoid receptors are sufficient for the negative feedback at the pituitary and hypothalamic level. On the other hand, high glucocorticoid receptor levels may be permissive for positive inputs to CRH neurons in the PVN.

Hypothalamic CRH Receptors. An important recent discovery of this laboratory was that stress induces CRH receptors in the PVN, suggesting that CRH has an autoregulatory effect in the CRH neuron. Studies during the past year using pharmacological blockade of CRH receptors in the brain support this hypothesis. We found that icv injection of a CRH antagonist reduces the elevation in plasma catecholamines and the increases in CRH mRNA in the PVN normally observed after stress, suggesting that central CRH exerts a positive feedback on CRH expression. Studies are in progress to determine whether CRH receptors in the PVN are responsible for this effect.

DEVELOPMENTAL ENDOCRINOLOGY BRANCH

Chief
Carolyn A. Bondy, M.D.

Senior Investigators
Greti Aguilera, M.D.
George P. Chrousos, M.D.
Gordon B. Cutler, M.D.
Lynnette K. Nieman, M.D.

Scientist Emeritus
Roy Hertz, M.D., Ph.D.

Tenure-Track Investigators
Domenico Accili, M.D.
Jeffrey Baron, M.D.

Staff Physicians
Lawrence Nelson, M.D.
Dimitris Papanicolaou, M.D.

Staff Scientist
Constantine Stratakis, M.D.

Senior Fellows
Veronica Abad, M.D.
Oluyemisi Adesanya, M.D.
Simon Bruce, M.D.
Michael Collins, M.D.
Penelope Feuillan, M.D.
Rachel Gafni, M.D.
Rhonda Hearns, M.D.
Jane Kim, M.D.
Lawrence Kirschner, M.D., Ph.D.
Mark Leondires, M.D.
Ellen Leschek, M.D.
Deborah Merke, M.D.
Zsolt Orban, M.D.
Kristina Rother, M.D.
Domenica Rubino, M.D.
James Segars, M.D.
Zhi-Bin Tong, M.D.
David Torpy, M.D.
Stacy Weil, M.D.
Martina Weise, M.D.
Karen Winer, M.D.
Jian Zhou, M.D., Ph.D.

Post-Doctoral Fellows
Clara Cheng, M.D.
Giovanni Di Cola, M.D.
Francesco De Luca, M.D.
Paul Driggers, Ph.D.
Sophia Kalantaridou, M.D.
Yoshiaki Kido, M.D.
Tomoshige Kino, M.D., Ph.D.
Xin-Ming Ma, Ph.D.
Edna Mancilla, M.D.
Maria Mericq, M.D.
Jan Nakae, M.D., Ph.D.
Sui Ng, M.D.
Tomasz Ochedalski, M.D., Ph.D.
Byung-Chui Park, Ph.D.
Johann Piquion, M.D.
Cristina Rabadan-Diehl, Ph.D.
Christina Samanthanam, M.D.
Toshiro Tajima, M.D., Ph.D.
Susan Taymans, Ph.D.
Elizabeth Webster, Ph.D.
Katrin Weichold, M.D., Ph.D.

Guest Researchers
Val Abbassi, M.D.
Stefan Bornstein, M.D., Ph.D.
Fernando Cassorla, M.D.
Frank Czerwiec, M.D., Ph.D.
Frank Chang, M.D.
Giovanni Cizza, M.D., Ph.D.
Helen Dichek, M.D.
Lorah Dorn, Ph.D.
Ilia Elenkov, M.D., Ph.D.
Denis Franchimont, M.D.
John Frattarelli, M.D.
Beth Hartog, M.D.
Hiroko Kanno, M.D.
Michael Karl, M.D.
Karen Oerter Klein, M.D.
Davide Lauro, M.D.
Carlos Longui, M.D., Ph.D.
Bradley Miller, M.D.
Gilbert Mottala, M.D.
Judy Novosad, M.D.
Susan B. Nunez, M.D.
Karel Pacak, M.D., Ph.D.
Owen Rennert, M.D.
Susan Rose, M.D.
Judith Ross, M.D.
Michael Sharon, M.D.
Pamela Stratton, M.D.
Thomas Temple, M.D.
Donna Vogel, M.D.
Alessandra Vottero, M.D.
Zhi-Bin Tong, M.D.
Fan Wei, M.D.
Jack Yanovski, M.D., Ph.D.

Pre-Doctoral Fellows
Marsha Bievre
George Joncas
Lawrence Kemp
Angela Lotsikas
Maria Slight
Jennifer Uyeda
Lora Wilson
Keith Vendola

ANNUAL BIBLIOGRAPHY

Accili D. Insulin receptor knock-out mice. Trends Endocrinol Metab 1997;8:101-104.

Accili D, Fishburn CS, Drago J, Steiner H, Lachowicz JE, Park BH, Gauda EB, Lee EJ, Cool MH, Sibley DR, Gerfen CR, Westphal H, Fuchs S. A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. Proc Natl Acad Sci USA 1996;93:1945-9.

Accili D, Fuchs S. A new look at dopamine D3 receptors [news]. Mol Psychiatry 1996;1:93-4.

Adashi EY, Resnick CE, Payne DW, Rosenfeld RG, Matsumoto T, Hunter MK, Gargosky SE, Zhou J, Bondy CA. The mouse intraovarian IGF-I system: departures from the rat paradigm. Endocrinology 1997;138:3881-90.

Aguilera G, Kiss A, Lu A, Camacho C. Regulation of adrenal steroidogenesis during chronic stress. Endocrine Res 1996;22:433-43.

Aguilera G, Jessop DS, Kiss A, Lightman SL. Differential regulation of hypothalamic pituitary corticotropin releasing hormone receptors during development of adjuvant-induced arthritis in the rat. J Endocrinol 1997;153:185-91.

Aguilera G, Rabadan-Diehl C, Luo X and Kiss A. Regulation of pituitary ACTH secretion during stress: role of corticotropin releasing hormone and vasopressin receptors. In: McCarty R. Aguilera G, Sabbou E. Kuetmansky R. eds. Stress: molecular genetic and neurobiological advances. New York, Gordon and Breach 1996;385-99.

Andersson S, Geissler WM, Wu L, Davis DL, Grumbach MM, New MI, Schwarz HP, Blethen SL, Mendonca BB, Bloise W, Witchel SF, Cutler GB Jr, Griffin JE, Wilson JD, Russel DW. Molecular genetics and pathophysiology of 17 beta-hydroxysteroid dehydrogenase 3 deficiency. J Clin Endocrinol Metab 1996;81:130-6.

Arnhold IJP, Latronico AC, Batista MC, Carvalho FM, Chrousos GP, Mendonca BB. Ovarian resistance to luteinizing hormone: a novel cause of amenorrhea and infertility. Fertil Steril 1997;67:394-7.

Avgerinos PC, Nieman LK, Oldfield EH, Cutler GB Jr. A comparison of the overnight and the standard metyrapone test for the differential diagnosis of Cushing syndrome. Clin Endocrinol 1996;45:483-91.

Avila NA, Premkumar A, Shawker TH, Jones JV, Laue L, Cutler GB Jr. Testicular adrenal rest tissue in congenital adrenal hyperplasia: gray scale and color Doppler ultrasound. Radiology 1996;198:99-104.

Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity. Endo Rev 1996;17:221-4.

Bamberger CM, Bamberger A, Wald M, Chrousos GP, Schulte HM. Inhibition of mineralocorticoid activity by the β-isoform of the human glucocorticoid receptor. J Steroid Biochem and Mol Biol 1997;60:43-50.

Barnes KM, Dickstein B, Cutler GB Jr, Fojo AT, Bates SE. The accumulation of steroids in multidrug-resistant cells and steroid antagonism of the P-glycoprotein drug efflux pump can be decreased by the PKC inhibitor, calphostin C. Biochemistry 1996;35:4820-7.

Baron J. Longitudinal bone growth: experimental methods. In: Thomas JA, ed. Endocrine methods. San Diego: Academic Press Inc: 1996;81-8.

Bruening JC, Winnay J, Bonner-Weir S, Taylor, SI, Accili D, Kahn CR. Development of a novel polygenic model of non-insulin-dependent diabetes mellitus in mice heterozygous for *IR* and *IRS*-1 null alleles. Cell 1997;88:561-72.

Brunetti A, Brunetti L, Foti D, Accili D, Goldfine ID. Human diabetes associated with defects in nuclear regulatory proteins for the insulin receptor gene. J Clin Invest 1996;97:258-62.

Burgess ES, Dorn LD, Haaga D, Chrousos GP. Sociotropy, autonomy, stress and depression in Cushing syndrome. J Nerv Ment Dis 1996;184:362-7.

Chin E, Zamah AM, Landau D, Gronbeck H, Flyvberg A, LeRoith D, Bondy CA. Changes in facilitative glucose transporter RNA levels in the diabetic rat kidney. Endocrinology 1997;138(3):1267-75.

Chrousos GP. Neuroendocrine and immune responses to stress in aging. Aging (Milano) 1997;9:25-40.

Chrousos GP. Organization and integration of the endocrine system. In: Sperling M, ed. Pediatric endocrinology. Philadelphia: WB Saunders, 1996;1-14.

Chrousos GP. The future of pediatric and adolescent endocrinology. Ann NY Acad Sci 1997;816:4-8.

Chrousos GP. The neuroendocrinology of stress: its relation to the hormonal milieu, growth, and development. Growth Genet Horm 1997;13:1-8.

Chrousos GP, Castro M, Leung DYM, Webster E, Kino T, Bamberger CM, Elliot S, Stratakis C, Karl M. Molecular mechanisms of glucocorticoid resistance/hyerpsensitivity. Am J Resp Crit Care Med 1996;154:S39-44.

Cizza G, Brady LS, Esclapes M, Blackman MR, Gold PW, Chrousos GP. Age and gender influence basal and stress-modulated hypothalamic-pituitary-thyroidal function in Fischer 344/N rats. Neuroendocrinology 1996;64:440-8.

Cizza G, Chrousos GP. Adrenocorticotropic hormone-dependent Cushing's syndrome. Cancer Treat Res 1997;89:25-40.

Cizza G, Chrousos GP. Anxiety and short stature. [Letter to the EditorJ. Pediatrics 1997;99:500.

Cizza G, Gold PW, Chrousos GP. High-dose transdermal estrogen, corticotropin-releasing hormone and postnatal depression. [Letter to the Editor]. J Clin Endocrinol Metab 1997;82:704.

Cizza G, Lotsikas AJ, Licinio J, Gold PW, Chrousos GP. Plasma leptin levels do not change in patients with Cushing's disease shortly after correction of hypercortisolism. J Clin Endocrinol Metab 1997;82:2747-50.

Cizza G, Nieman LK, Doppman JL, Passaro MD, Czerwiec FS, Chrousos GP, Cutler GB Jr. Factitious Cushing syndrome. J Clin Endocrinol Metab 1996;81:3573-7.

Creatsas G, Mastorakos G, Chrousos GP, eds. Adolescent gynecology and endocrinology: Basic and clinical aspects, vol 816. New York: Ann NY Acad Sci, 1997;470.

Crofford LJ, Kalogeras KT, Mastorakos G, Magiakou M-A, Wells J, Kanik KS, Gold PW, Chrousos GP, Wilder RL. Circadian relationships between interleukin (IL)-6 and hypothalamic-pituitary-adrenal axis hormones: failure of IL-6 to cause sustained hypercortisolism in patients with early untreated rheumatoid arthritis. J Clin Endocrinol Metab 1997;82:1279-83.

Cutler GB Jr. Ambiguous genitalia. In: Hurst JW ed. Medicine for the Practicing Physician, 4th ed. Stamford: Appleton Lange, 1996;617-23.

Cutler GB Jr. Precocious puberty. In: Hurst, JE, ed. Medicine for the Practicing Physician, 4th ed. Stamford: Appleton Lange 1996;62-8.

Cutler GB Jr. The role of estrogen in bone growth and maturation during childhood and adolescence. J Steroid Biochem Mol Biol 1997;61:141-4.

Cutler GB Jr. Treatment of congenital adrenal hyperplasia: the case for new medical approaches. J Clin Endocrinol Metab 1996;81:3180-90.

Czerwiec FS, Cutler GB Jr. Modern approach to corticosteroid replacement. Curr Opinion Endocrinol Diabetes 1996;3:239-46.

de Castro M, Chrousos GP. Glucocorticoid resistance. Curr Ther Endocrinol Metab 1997;6:188-9.

de Lange P, Koper JW, Huizenga NATM, Brinkman AO, de Jong FH, Cato ACB, Karl M, Chrousos GP, Lamberts SWJ. Differential hormone-dependent transactivation and transrepression by naturally occurring human glucocorticoid receptor (GR) variants. Mol Endocrinol 1997;11:1156-64.

De Luca F, Ray K, Mancilla EE, Fan GF, Winer KK, Gore P, Spiegel AM, Baron J. Sporadic hypoparathyroidism caused by de novo gain-of-function mutations of the Ca^{2+}-sensing receptor. J Clin Endocrinol Metab 1997;82:2710-15.

DeMarco L, Stratakis CA, Boson WL, Jakobovitz O, Carson E, Andrade LM, Amaral VF, Rocha JL, Chrousos GP, Nordenskjöld M, Friedman E. Sporadic cardiac myxomas and tumors from patients with Carney complex are not associated with activating mutations of the Gsα gene. Hum Genet 1996;98:185-8.

Di Cola G, Cool MH, Accili D. Hypoglycemic effect of IGF-1 mice lacking insulin receptors. J Clin Invest 1997;99:2538-44.

Dorn LD, Burgess ES, Dichek HL, Putnam F, Chrousos GP, Gold PW. Thyroid hormone concentrations in depressed and non-depressed adolescents: group differences and behavioral relations. J Am Acad Child Adolesc Psychiatry 1996;35:299-306.

Dorn LD, Burgess ES, Friedman TC, Dubbert B, Gold PW, Chrousos GP. The longitudinal course of psychopathology in Cushing syndrome after correction of hypercortisolism. J Clin Endocrinol Metab 1997;82:912-9.

Dorn LD, Burgess ES, Susman EEJ, Von Eye A, DeBellis MD, Gold PW, Chrousos GP. Response to ovine corticotropin releasing hormone in depressed and nondepressed adolescents: Does gender make a difference? J Am Acad Child Adolesc Psychiatry 1996;35:764-73.

Dorn LD, Chrousos GP. The neurobiology of stress: understanding regulation of affect during female biological transitions. Semin Reprod Endocrinol 1997;15:29-45.

Elenkov IJ, Papanicolaou DA, Wilder RL, Chrousos GP. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. Proc Assn Am Phys 1996;108:374-81.

Flack MR, Chrousos GP. Neoplasms of the adrenal cortex. In: Holland R, et al., eds. Cancer medicine. New York: Lead and Fibinger, 1996;1563-70.

Friedman TC, Mastorakos G, Newman TD, Mullen NM, Horton EG, Costello R, Papadopoulos NM, Chrousos GP. Carbohydrate and lipid metabolism in endogenous hypercortisolism: shared features with metabolic syndrome X and noninsulin-dependent diabetes mellitus. Endocr J 1996;43:645-56.

Friedman TC, Nieman LK, Yanovski JA, Doppman JL, Cutler GB Jr., Oldfield EH, Gold PW, Chrousos GP, Kalogeras KT. Inferior petrosal sinus arginine vasopressin concentrations in normal volunteers and patients with Cushing disease. J Clin Endocrinol Metab 1996;81:3068-72.

Ghizzoni L, Mastorakos G, Street ME, Vottero A, Mazzardo G, Vanelli M, Chrousos GP, Bernasconi S. Spontaneous thyrotropin and cortisol secretion interactions in patients with nonclassical 21-hydroxylase deficiency and control children. J Clin Endocrinol Metab 1997;82:3677-83.

Ghizzoni L, Mastorakos G, Vottero A, Barreca A, Furlini M, Cesarone A, Ferrari B, Chrousos GP, Bernasconi S. Corticotropin-releasing hormone (CRH) inhibits steroid biosynthesis by cultured human granulosa-lutein cells in a CRH and interleukin-1 receptor-mediated fashion. Endocrinology 1997;138:4806-11.

Ghizzoni L, Mastorakos G, Vottero A, Magiakou MA, Chrousos GP, Bernasconi S. Spontaneous cortisol and growth hormone secretion interactions in patients with nonclassic 21-hydroxylase deficiency (NCCAH) and control children. J Clin Endocrinol Metab 1996;81:482-7.

Gold PW, Wong M-L, Chrousos GP, Licinio J. Stress system abnormalities in melancholic and atypical depression: molecular, pathophysiological, and therapeutic implications. [EditorialJ. Mol Psychiatry 1996;1:257-64.

Gordeladze JO, Høvik KE, Merendino JJ, Hermouet S, Gutkind S, Accili D. Effect of activating and inactivating mutations of G_s- and G_{i2}-alpha protein subunits on growth and differentiation of 3T3-L1 preadipocytes. J Cell Biochem 1997;64:242-57.

Heinrichs C, Colli MJ, Yanovski JA, Laue L, Gerstl NA, Kramer AD, Uyeda JA, Baron J. Effects of fasting on the growth plate: systemic and local mechanisms. Endocrinology 1997;138:5359-65.

Hirschfeld S, Laue L, Cutler GB Jr, Pizzo PA. Thyroid abnormalities in children infected with human immunodeficiency virus. J Pediatr 1996;128:704.

Jezova D, Kiss A and Aguilera G. Studies on the mechanisms of the atypical plasma vasopressin responses to stress in chronically adrenal demedullated rats. Stress 1996;1:73-81.

Johnson EO, Kamilaris TC, Calogero AE, Gold PW, Chrousos GP. Effects of early parenting on growth and development in a small primate. Pediatr Res 1996;39:999-1005.

Johnson EO, Decker L, Gold PW, Chrousos GP. Distribution of hippocampal mineralocorticoid and glucocorticoid receptor mRNA in a glucocorticoid resistant nonhuman primate. Steroids 1996;61:69-73.

Johnson EO, Kamilaris TC, Carter CS, Calogero AE, Gold PW, Chrousos GP. The biobehavioral consequences of psychogenic stress in a small, social primate (*Callithrix jacchus jacchus*). Biol Psychiatry 1996;40:317-37.

Jose PA, Drago J, Accili D, Eisner GM, Felder RA. Transgenic mice to study the role of dopamine receptors in cardiovascular function. Clin Exp Hypertens 1997;19:15-25.

Jui HY, Accili D, Taylor SI. Characterization of a hybrid receptor formed by dimerization of the insulin receptor-related receptor (IRR) with the insulin receptor (IR): coexpression of cDNAs encoding human IRR and human IR in NIH-3T3 cells. Biochemistry 1996;35:14326-30.

Kalogeras KT, Nieman LK, Friedman TC, Doppman JL, Cutler GB, Chrousos GP, Wilder RL, Gold PW, Yanovski JA. Inferior petrosal sinus sampling in normal human subjects reveals a unilateral corticotropin-releasing hormone-induced arginine vasopressin release associated with ipsilateral adrenal corticotropin secretion. J Clin Invest 1996;97:2045-50.

Karl M, Chrousos GP. Experimental and clinical models of CRH-induced pituitary tumors. In: Melmed S, ed. Oncogenesis and molecular biology and pituitary tumors. Basel: Karger, 1996;54-71.

Karl M, Lamberts SWJ, Koper JW, Katz DA, Huizenga NE, Kino T, Haddad BR, Hughes MR, Chrousos GP. Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assoc Amer Physicians 1996;108:296-307.

Karl M, Saviolakis GA, Gravanis A, Chrousos GP, Margioris AN. The PC 12 rat pheochromocytoma cell line expresses the prodynorphin gene and secretes the 8 kdalton dynorphin product. Regul Pept 1996;61:99-104.

Karl M, Von Wichert G, Kempter E, Katz DA, Reincke M, Monig H, Ali IU, Stratakis CA, Oldfield EH, Chrousos GP, Schulte HM. Nelson's syndrome associated with somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab 1996;81:122-9.

Kim TJ, Anasti JN, Flack MR, Kimzey LM, Defensor RA, Nelson LM. Routine endocrine screening for patients with karyotypically normal spontaneous premature ovarian failure. Obstet Gynecol 1997;89:777-9.

Klein KO, Cutler GB Jr. The hypothalamus and neuroendocrine disorders. In: Berg BO, ed. Principles of child neurology. New York: McGraw-Hill, 1996;437-57.

Klein KO, Cutler GB Jr. Puberty. In: Hillier SG, Kitchener H, Nielson JP, eds. Scientific essentials of reproductive medicine. London: W.B. Saunders 1996;196-207.

Latronico AC, Anasti J, Arnhold IJP, Rapaport R, Mendonca BB, Bloise W, de Castro M, Tsigos C, Chrousos GP. Testicular and ovarian resistance to luteinizing hormone caused by homozygous inactivating mutations of the luteinizing hormone receptor gene. N Engl J Med 1996;334:507-12.

Latronico AC, Chrousos GP. Adrenocortical tumors (Extensive personal experience article). J Clin Endocrinol Metab 1997;82:1317-24.

Latronico AC, Chrousos GP. Neoplasms of the adrenal cortex. Clinical and basic aspects. Cancer Treat Res 1997;89:217-37.

Laue L, Wu SM, Kudo M, Bourdony CJ, Cutler GB, Hsueh AJW, Chan WY. Compound heterozygous mutations of the luteinizing hormone receptor gene in Leydig cell hypoplasia. Mol Endocrinol 1996;10:987-97.

Laue L, Wu SM, Kudo M, Hsueh AJW, Cutler GB, Jelly DH, Chan WY. Heterogeneity of activating mutations of the human luteinizing hormone receptor in male-limited precocious puberty. Biochem Mol Med 1996;58:192-8.

Laue L, Merke DP, Jones JV, Barnes KM, Hill SV, Cutler GB. A preliminary study of flutamide, testolactone, and reduced hydrocortisone dose in the treatment of congenital adrenal hyperplasia. J Clin Endocrinol Metab 1996;81:3535-9.

Leong GM, Mercado-Asis LB, Reynolds JC, Oldfield EH, Chrousos GP. The effect of Cushing disease on bone mineral density, body composition, growth and puberty: a report of an identical adolscent twin pair. J Clin Endocrinol Metab 1996;81:1905-11.

Leung DYM, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, Chrousos GP, Klemm DJ. Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. J Exp Med 1997;186:1567-74.

Leschek EW, Cutler GB. Familial male precocious puberty. Curr Ther Endocrinol Metab 1997;6:343-5.

Leschek EW, Doppman JL, Pass HI, Cutler GB. Clinical Case Seminar: Localization by venous sampling of occult chorionic gonadotropin-secreting tumor in a boy with mosaic Klinefelter syndrome and precocious puberty. J Clin Endocrinol Metab 1996;81:3825-8.

Licinio J, Gold PW, Chrousos GP, Sternberg EM. Forty years of neuroendocrinology: a tribute to SM McCann [editorial]. Mol Psychiatry 1997;2:347-9.

Licinio J, Mantzoros C, Negrão AB, Cizza G, Wong M-L, Bongiorno PB, Chrousos GP, Karp B, Allen C, Flier JS, Gold PW. Human leptin levels are pulsatile and inversely related to pituitary-adrenal function. Nat Med 1997;3:575-9.

Louvi A, Accili D, Efstratiadis A. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev Biol 1997;189:33-48.

Magiakou MA, Chrousos GP. Corticosteroid therapy, nonendocrine disease, and corticosteroid withdrawal. Curr Ther Endocrinol Metab 1997;6:138-42.

Magiakou MA, Chrousos GP. Glucocorticoid therapy and withdrawal. In: Bone RC, ed. Current practice of medicine. Philadelphia: Current Medicine, Inc., 1996;6.1-6.6.

Magiakou MA, Mastorakos G, Chrousos GP. Cushing syndrome: differential diagnosis and treatment. In: Wierman ME, ed. Diseases of the pituitary: diagnosis and treatment. Totowa: Humana Press, 1997;179-202.

Magiakou MA, Mastorakos G, Webster EL, Chrousos GP. The hypothalamic-pituitary-adrenal axis and the female reproductive system. Ann NY Acad Sci 1997;816:27-41.

Magiakou MA, Mastorakos G, Zachman K, Chrousos GP. Blood pressure in childen and adolescents with Cushing syndrome before and after surgical cure. J Clin Endocrinol Metab 1997;82:1734-8.

Magiakou MA, Mastorakos G, Rabin D, Dubbert B, Gold PW, Chrousos GP. Hypothalamic corticotropin releasing hormone suppression during the postpartum period: implications for the increase of psychiatric manifestations during this time. J Clin Endocrinol Metab 1996;81:1912-7.

Magiakou MA, Mastorakos G, Webster E, Chrousos GP. The hypothalamic-pituitary-adrenal axis and the female reproductive system. Ann NY Acad Sci 1997;816:27-41.

Magiakou MA, Mastorakos G, Rabin D, Margioris AN, Dubbert 8, Calogero AE, Tsigos C, Munson PJ, Chrousos GP. The maternal hypothalamic-pituitary-adrenal axis in third trimester human pregnancy. Clin Endocrinol (Oxf) 1996;44:419-28.

Maity R, Caspi RR, Nair S, Rizzo LV, Nelson LM. Murine post-thymectomy autoimmune oophoritis develops in association with a persistent neonatal-like Th2 response. Clin Immunol Immunopathol 1997;83:230-6.

Maity R, Nair SR, Caspi RR, Nelson LM. Post-thymectomy murine experimental autoimmune oophoritis is associated with reduced natural killer cell activity. Am J Reprod Immunol 1997;38:360-5.

Makrigiannakis A, Zoumakis E, Margioris AN, Stournaras C, Chrousos GP, Gravanis A. Regulation of the promoter of the human corticotropin-releasing hormone (CRH) gene in transfected human endometrial cells. Neuroendocrinology 1996;64:85-92.

Mancilla EE, De Luca F, Ray K, Winer KK, Fan GF, Baron J. A Ca^{2+}-sensing receptor mutation causes hypoparathyroidism by increasing receptor sensitivity to Ca^{2+} and maximal signal transduction. Pediatr Res 1997;42:443-7.

Masi AT, Chrousos GP. Dilemmas of low-dosage glucocorticoid therapy in rheumatoid arthritis: considerations of timing (editorial). Ann Rheum Dis 1997;56:1-4.

Masi AT, Chrousos GP. The hypothalamic-pituitary-adrenal-glucocorticoid axis in rheumatoid arthritis (editorial). J Rheumatol 1996;23:577-81.

Mason RB, Nieman LK, Doppman JL, Oldfield EH. Selective excision of adenomas originating in or extending into the pituitary stalk with preservation of pituitary function. J Neurosurg 1997;87:343-51.

Mastorakos G, Scopa CD, Kao LC, Vryonidou A, Friedman TC, Kattis D, Phenekos C, Rabin D, Chrousos GP. Presence of immunoreactive corticotropin releasing-hormone (CRH) in human endometrium. J Clin Endocrinol Metab 1996;81:1046-50.

Mastorakos G, Ghizzoni L, Webster EL, Chrousos GP. Autocrine-paracrine role of ovarian corticotropin-releasing hormone. Ann NY Acad Sci 1997;816:22-6.

Maunsbach AB, Marples D, Chin E, Ning G, Bondy C, Agre P, Nielsen S. Aquaporin-1 water channel expression in human kidney. J Am Soc Nephrol 1997;8:1-14.

Mercado-Asis LB, Yanovski JA, Tracer HL, Chik CL, Cutler GB. Acute effects of bromocryptine, cyproheptadine, and valproic acid on plasma adrenocorticotropin secretion in Nelson's syndrome. J Clin Endocrinol Metab 1997;82:514-7.

Merke DP, Cutler GB. New approaches to the treatment of congenital adrenal hyperplasia. JAMA 1997; 277:1073-6.

Merke DP, Cutler GB. The evaluation and management of precocious puberty. Arch Dis Child 1996;75:269-71.

Michelson D, Galliven E, Hill L, Demitrack M, Chrousos GP, Gold PW. Chronic imipramine is associated with diminished hypothalamic-pituitary-adrenal axis responsitivity in healthy humans. J Clin Endocrinol Metab 1997;82:2601-6.

Michelson D, Stratakis C, Hill LY, Reynolds J, Galliven E, Chrousos GP, Gold PW. Bone mineral density is decreased in women with depression. N Engl J Med 1996;335:1176-81.

Moy E, Kimzey LM, Nelson LM, Blithe DL. Glycoprotein hormone α-subunit functions synergistically with progesterone to stimulate differentiation of cultured human endometrial stromal cells to decidualized cells: a novel role for free α-subunit in reproduction. Endocrinology 1996;137:1332-9.

Nair S, Caspi RR, Nelson LM. Susceptibility to murine experimental autoimmune oophoritis is associated with genes outside the major histocompatibility complex. Am J Reprod Immunol 1996;36:107-110.

Ng S, Zhou J, Adesanya O, Wang J, LeRoith D, Bondy C. Growth hormone treatment induces mammary gland hyperplasia in aging primates. Nat Med 1997;3:1141-4.

Nieman LK. Cushing's syndrome treatment. In: Bardin CW, ed. Current therapy of endocrinology and metabolism. Philadelphia: WB Saunders, 1996;609-14.

Nieman LK. Cushing's syndrome. Curr Ther Endocrinol Metab 1997;6:161-4.

Nunez SB, Blye RP, Thomas PM, Reel JR, Barnes KM, Malley JD, Cutler GB . Recovery of reproductive function in male and female rats treated with the potent aromatase inhibitor fadrozole hydrochloride. Reprod Toxicol 1996;10:373-7.

Papanicolaou DA. Cytokines and adrenal insufficiency. Curr Opin Endocrinol Diabetes 1997;4:194-8.

Papanicolaou DA, Petrides JS, Tsigos C, Bina S, Kalogeras KT, Wilder R, Gold PW, Deuster PA, Chrousos GP. Exercise stimulates interleukin-6 secretion: inhibition by glucocorticoids and correlation with catecholamines. Am J Physiol 1996;E601-5.

Papanicolaou DA, Tsigos C, Oldfield EH, Chrousos GP. Acute glucocorticoid deficiency is associated with plasma elevation of interleukin-6: does the latter participate in the symptomatology of the steroid withdrawal syndrome and adrenal insufficiency?. J Clin Endocrinol Metab 1996;81:2303-6.

Petrides JS, Gold PW, Mueller GP, Singh A, Stratakis CA, Chrousos GP, Deuster PA. Marked differences in functioning of the hypothalamic-pituitary-adrenal axis between groups of men. J Appl Physiol 1997;82:1979-88.

Petzke F, Heppner C, Mbulamberi D, Winkelmann W, Chrousos GP, Allolio B, Reincke M. Hypogonadism in Rhodesian sleeping sickness: evidence for acute and chronic dysfunction of the hypothalamic-pituitary-gonadal axis. Fertil Steril 1996;65:68-75.

Pillemer SR, Bradley LA, Crofford LJ, Moldofsky H, Chrousos GP. The neuroscience and endocrinology of fibromyalgia. Arthritis Rheum 1997;40:1928-39.

Premkumar A, Stratakis CA, Shawker TH, Papanicolaou DA, Chrousos GP. Testicular ultrasound in Carney complex: report of three cases. J Clin Ultrasound 1997;25:211-4.

Rabadan-Diehl C, Kiss A, Camacho C, Aguilera G. Regulation of messenger ribonucleic acid for corticotropin releasing hormone receptors in the pituitary during stress. Endocrinology 1996;137:3808-14.

Rabadan-Diehl C, Makara G, Kiss A, Lolait SJ, Zelena D, Ochedaski T, Aguilera G. Regulation of pituitary V1b vasopressin receptor mRNA by adrenalectomy and glucocorticoid administration. Endocrinology 1997;138:5189-5194

Rabadan-Diehl C, Makara G, Kiss A, Zelena D, Aguilera G. Regulation of pituitary corticotropin releasing hormone (CRH) receptor mRNA and CRH binding during adrenalectomy: role of glucocorticoids and hypothalamic factors. J Neuroendocrinol 1997;9:689-697.

Ramayya MS, Zhou J, Kino T, Segars JH, Bondy CA, Chrousos GP. Steroidogenic factor-1 RNA expression in steroidogenic and nonsteroidogenic human tissues: Northern blot and in situ hybridization studies. J Clin Endocrinol Metab 1997;82:1799-806.

Reincke M, Beuschlein F, Latronico A-C, Arlt W, Chrousos GP, Allolio B. Expression of adrenocorticotropic hormone receptor mRNA in human adrenocortical neoplasms: correlation with P450scc expression. Clin Endocrinol (Oxf) 1997;46:619-26.

Reincke M, Mora P, Beuschlein F, Arlt W, Chrousos GP, Allolio B. Deletion of the adrenocorticotropin receptor gene in human adrenocortical tumors: implications for tumorigenesis. J Clin Endocrinol Metab 1997;82:3054-8.

Reincke M, Wachenfeld C, Mora P, Thumser A, Jaursch-Hancke C, Abdelhamnid S, Chrousos GP, Allolio B. P53 mutations in adrenal tumors: caucasian patients do not show the exon 4 "hot spot" found in Taiwan. J Clin Endocrinol Metab 1996;81:3636-8.

Rose SR, Municchi G, Barnes KM, Cassorla FG, Cutler GB. Overnight growth hormone concentrations are usually normal in pubertal children with idiopathic short stature - a clinical research center study. J Clin Endocrinol Metab 1996;81:1063-8.

Ross JL, McCauley E, Roeltgen, Long L, Kushner H, Feuillan P, Cutler GB. Self-concept and behavior in adolescent girls with Turner syndrome: potential estrogen effects. J Clin Endocrinol Metab 1996;81:926-31.

Ross JL, Feuillan P, Kushner H, Roeltgen D, Cutler GB. Absence of growth hormone effects on cognitive function in girls with Turner syndrome. J Clin Endcorinol Metab 1997;82:1814-7.

Sarlis NJ, Chrousos GP, Doppman JL, Carney JA, Stratakis CA. Primary pigmented nodular adrenocortical disease: reevaluation of a patient with Carney complex 27 years after unilateral adrenalectomy. J Clin Endocrinol Metab 1997;82:1274-8.

Sartor O, Cutler GB. Treatment of Cushing's syndrome with the antiglucocorticoid mifepristone (RU486). Clin Obstet Gynecol 1996;39:506-10.

Schiffmann R, Tedeschi G, Kinkel RP, Trapp BD, Frank JA, Kaneski CR, Brady RO, Barton RO, Barton NW, Nelson L, Yanovski JA. Leukodystrophy in patients with ovarian dysgenesis. Ann Neurol 1997;41:654-61.

Stratakis CA, Carney A, Ping J, Papanicolaou DA, Karl M, Kastner DL, Pras E, Chrousos GP. Carney complex, a familial male neoplasia and lentiginosis syndrome: analysis of 11 kindreds and linkage to the short arm of chromosome 2. J Clin Invest 1996;97:699-705.

Stratakis CA, Chrousos GP. Endocrine tumors. In: Pizzo PA, Poplack DG, eds. Principles and practice of pediatric oncology. Philadelphia: J.B. Lippincott Raven Publishers, 1997;947-76.

Stratakis CA, Chrousos GP. Hypothalamic hormones: GnRH, TRH, GHRH, SRIF, CRH, and dopamine. In: Conn PM, Melmed S, eds. Endocrinology: basic and clinical principles. Totowa: Humana Press, 1997;185-209.

Stratakis CA, Chrousos GP. The familial lentiginosis syndromes: link to inherited neoplasias and identification of a genetic locus. Hellenic Med J 1997;1:15-9.

Stratakis CA, Chrousos GP. Transient elevation of serum thyroid hormone levels following lithium discontinuation. Eur J Pediatr 1996;155:939-41.

Stratakis CA, Courcoutsakis NA, Abati A, Filie A, Doppman JL, Carney JA, Shawker T. Thyroid gland abnormalities in patients with the syndrome of spotty skin pigmentation, myxomas, endocrine overactivity, and schwannomas (Carney complex). J Clin Endocrinol Metab 1997;82:2037-43.

Stratakis CA, Jenkins RB, Pras E, Mitsiadis CS, Raff SB, Stalboerger PG, Tsigos C, Carney A, Chrousos GP. Cytogenic and microsatellite alterations in tumors from patients with the syndrome of myxomas, spotty skin pigmentation, and endocrine overactivity (Carney complex). J Clin Endocrinol Metab 1996;81:3607-14.

Stratakis CA, Lin J-P, Pras E, Rennert OM, Bourdony CJ, Chan W-Y. Segretation of allgrove (triple A) syndrome in Puerto Rican kindres with chromosome 12 (12q13) polymorphic markers. Proc Assoc Am Physicians 1997;109:478-82.

Stratakis CA, Mitsiades NS, Chrousos GP, Margioris AN. Dopamine affects rat placenta opioids in a receptor- and opioid type-specific manner. Eur J Pharmacol 1996;315:53-8.

Stratakis CA, Orban Z, Burns AL, Vottero A, Mitsiades CS, Marx SJ, Abbassi V, Chrousos GP. Dideoxyfingerprinting (ddF) analysis of the type X collagen gene (COL10A1) and identification of a novel mutation (S671P) in a kindred with Schmid metaphyseal chondrodysplasia. Biochem Mol Med 1996;59:112-7.

Stratakis CA, Sarlis NJ, Berrettini WH, Badner JA, Chrousos GP, Gershon ES, Detera-Wadleigh SD. Lack of linkage between the corticotropin-releasing hormone (CRH) gene and bipolar affective disorder. Mol Psychiatry 1997;2:483-5.

Susman E, Dorn L, Inoff-Germain G, Nottelmann E, Chrousos GP. Cortisol reactivity, distress behavior, and behavioral and psychological problems in young adolescents: a longitudinal perspective. J Res Adolescence 1997;7:81-105.

Tajima T, Fujieda K, Nakae J, Toyoura T, Shimozawa K, Kusuda S, Goji K, Nagashima T, Cutler GB Jr. Molecular basis of nonclassical steroid 21-hydroxylase deficiency detected by neonatal mass screening in Japan. J Clin Endocrinol Metab 1997;82:2350-6.

Taymans SE, DeVries AC, DeVries MB, Nelson RJ, Friedman TC, Castro M, Detera-Wadleigh S, Carter CS, Chrousos GP. The hypothalamic-pituitary axis of the prairie vole (*Microtus ochrogaster*): evidence for target tissue glucocorticoid resistance. Gen Comp Endocrinol 1997;106:48-61.

Torpy DJ, Chrousos GP. General adaptation syndrome: an overview. In: Ober KP, ed. Endocrinology of critical disease. Totowa: Humana Press, 1997;1-24.

Torpy DJ, Chrousos GP. The three-way interactions between the hypothalamic-pituitary-adrenal and gonadal axes and the immune system. Ballière's Clin Rheumatol 1996;10:181-98.

Torpy D, Papanicolaou DA, Chrousos GP. Sexual dimorphism of the human stress response may be due to estradiol-mediated stimulation of hypothalamic corticotropin-releasing hormone synthesis (Letter to the editor). J Clin Endocrinol Metab 1997;82:982.

Tsigos C, Chrousos GP. Differential diagnosis and management of Cushing's syndrome. Annu Rev Med 1996;47;443-61.

Tsigos C, Kamilaris T, Chrousos GP. Adrenal diseases. In: Moore WT, Eastman R, eds. Diagnostic endocrinology. Toronto: B.C. Decker, 1996;123-56.

Tsigos C, Latronico C, Chrousos GP Luteinizing hormone resistance syndromes. Ann N Y Acad Sci 1997;816:263-73.

Tsigos C, Papanicolaou DA, Defensor R, Mitsiadis CS, Kyrou I, Chrousos GP. Dose effects of recombinant human interleukin-6 on pituitary hormone secretion and energy expenditure. Neuroendocrinology 1997;66:54-62.

Tullio AN, Accili D, Ferrans VJ, Yu ZX, Takeda K, Grinberg A, Westphal, H, Preston YA, Adelstein, RS. Nonmuscle myosin II-B is required for normal development of the mouse heart. Proc Natl Acad Sci USA 1997;94:12407-12.

Van Wyk JJ, Gunther DF, Ritzen EM, Wedell A, Cutler GB Jr, Migeon CJ. The use of adrenalectomy as a treatment for congenital adrenal hyperplasia. J Clin Endocrinol Metab 1996;81:3180-90.

Vgontzas AN, Bixler EO, Papanicolaou DA, Kales A, Stratakis CA, Vela-Bueno A, Gold PW, Chrousos GP. REM sleep correlates with the overall activities of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic system in healthy humans. J Clin Endocrinol Metab 1997;82:3278-80 .

Vgontzas AN, Papanicolaou DA, Bixler EO, Kales A, Tyson K, Chrousos GP. Elevation of plasma cytokines in disorders of excessive daytime sleepiness: role of sleep disturbance and obesity. J Clin Endocrinol Metab 1997;82:1313-6.

Walther MM, Cutler GB. Urodynamic abnormalities in two brothers with adrenomyeloneuropathy. World J Urol 1997;15:262-5.

Webster EL, Elenkov IJ, Chrousos GP. Corticotropin-releasing hormone acts on immune cells to elicit pro-inflammatory responses. Mol Psychiatry 1997;2:345-6.

Webster EL, Elenkov IJ, Chrousos GP. The role of corticotropin-releasing hormone in neuroendocrine-immune interactions. Mol Psychiatry 1997;2:368-72.

Webster EL, Lewis DB, Torpy DJ, Zachman EK, Rice KC, Chrousos GP. *In vivo* and *in vitro* characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation. Endocrinology 1996;137:5747-50.

White PC, Pescovitz OH, Cutler GB Jr. Synthesis and metabolism of corticosteroids. In: Becker KL, ed. Principles and practice of endocrinology and metabolism, 2nd ed. New York: JB Lippincott, 1996;647-62.

Winer KK, Yanovski JA, Cutler GB. Synthetic human parathyroid hormone 1-34 vs calcitriol and calcium in the treatment of hypoparathyroidism. JAMA 1996;276:631-6.

Wong M, Ramayya MS, Chrousos GP, Driggers PH, Parker KL. Cloning and sequencing analysis of the human gene encoding steroidogenic factor 1. J Mol Endocrinol 1996;17:139-47.

Yano J, Kohn LD, Saji M, Kataoka N, Okuno A, Cutler GB. A case of male-limited precocious puberty caused by a point mutation in the second transmembrane domain of the luteinizing hormone/choriogonadotropin receptor gene. Biochem Biophys Res Commun 1996;220:1036-42.

Yanovski JA, Cutler GB. The reproductive axis: pubertal activation. In: Adashi EY, Rock JA, and Rosenwaks Z, eds. Reproductive endocrinology, surgery, and technology. New York, Raven Press, 1996;196-207.

Yanovski JA, Friedman TC, Nieman LK, Chrousos GP, Cutler GB Jr, Doppman JL, Kalogeras KT. Inferior petrosal sinus AVP in patients with Cushing's syndrome. Clin Endocrinol (Oxf) 1997;47:199-206.

Yanovski JA, Yanovski SZ, Cutler GB, Chrousos GP, Filmer K. Differences in the hypothalamic-pituitary-adrenal axis of African American and Caucasian girls. J Pediatr 1996;129:130-6.

Yanovski JA, Yanovski SZ, Filmer K, Hubbard VS, Avila N, Lewis B, Reynolds J, Flood M. Differences in body composition of Black and White prepubertal girls. Am J Clin Nutr 1996;64:833-9:.

Yanovski JA, Yanovski SZ, Friedman TC, Loh YP, Jayasvasti V, Cutler GB, Chrousos GP. Etiology of the differences in corticotropin-releasing hormone-induced ACTH secretion of black and white women. J Clin Endocrinol Metab 1996;81:3307-11.

Yanovski JA, Yanovski SZ, Gold PG. Chrousos GP. Differences in corticotropin-releasing hormone-stimulated ACTH and cortisol before and after weight loss. J Clin Endocrinol Metab 1997;82:1874-8.

Zhou J, Adesanya OO, Vatzias G, Hammond JM, Bondy CA. Selective expression of IGF system during porcine ovary follicular selection. Endocrinology 1996;137:4893-4901.

ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

Kevin J. Catt, M.D., Ph.D., Chief

The research programs of the Endocrinology and Reproduction Research Branch (ERRB) are directed at the elucidation of molecular and cellular mechanisms involved in hormone secretion and action. Major topics of interest include: the characterization of peptide hormones and their mechanisms of action in endocrine target cells; the structure-function relationships of peptide and glycoprotein hormones; and the regulation of hormone biosynthesis and secretion. At the molecular level, research is performed on the plasma-membrane receptors and intracellular signaling processes that are responsible for the control of secretory responses, differentiation, and metabolic regulation in endocrine target cells. The ERRB investigators share interests in the mechanisms of action of peptide and glycoprotein hormones, the role of neuropeptides in hypothalamic-pituitary and gonadal regulation, the control of gonadal and adrenal function by pituitary hormones, the renin-angiotensin system and aldosterone secretion, and the mechanisms and roles of protein phosphorylation in metabolic regulation and signal transduction.

PEPTIDE HORMONES AND ENDOCRINE REGULATION

The Section on Hormonal Regulation, directed by ***Kevin Catt***, performs research on the mechanisms by which peptide hormones control the activities of endocrine and other target cells. This includes the characterization of receptors, signal transduction pathways, and other cellular processes involved in the neural control of gonadotropin-releasing hormone (GnRH) biosynthesis and secretion, the regulation and structure-function properties of receptors for GnRH and angiotensin II (Ang II), and the effects of GnRH and Ang II on target cells in the hypothalamus, pituitary, and adrenal gland. Current research is focused on the structural features and signal transduction mechanisms of the GnRH and Ang II receptors, and their regulatory actions on the metabolic, secretory, and growth responses of hypothalamic, pituitary, and adrenal cells.

Structure-Function Properties of the GnRH Receptor. The hypothalamic decapeptide, GnRH, acts via its specific receptor in anterior pituitary gonadotrophs to regulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and is essential for the maintenance of normal reproductive function. The cDNAs for the GnRH receptors of mouse, rat, sheep, cow, dog, and human have been cloned, and their deduced amino acid sequences are more than 85% identical. The coding region of the GnRH receptor contains seven putative transmembrane domains (TM I-VII) as well as many of the conserved residues and sequences typical of other members of the G protein-coupled receptor (GPCR) superfamily. However, the GnRH receptor exhibits several unique features that include a long and highly basic first intracellular loop; the replacement of Tyr by Ser in the conserved DRY motif located at the cytoplasmic end of TM III, and the absence of a carboxyterminal cytoplasmic tail. In addition, the highly conserved Asp and Asn residues usually located in TM II and TM VII, respectively, are exchanged. Agonist binding to the GnRH receptor stimulates the rapid hydrolysis of phosphatidylinositol 1,4,5-trisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol, thereby mobilizing Ca^{2+} and activating protein kinase C to initiate a variety of cellular responses. Receptor activation is followed by desensitization, and by internalization and down-regulation of the receptor via endocytosis and processing.

The molecular mechanism(s) of GnRH receptor activation are being clarified by site-directed mutagenesis to identify residues and sequences that are involved in G protein activation, signal transduction, and receptor internalization. In previous studies on the structure-function relationships of the GnRH receptor, we found that a conserved Leu-147 residue located in the second intracellular loop is essential for both agonist-induced signal transduction and receptor internalization. In addition, the aromatic moiety of the Tyr-322 component of the DPLIY motif in the seventh transmembrane domain of the GnRH receptor was shown to be a critical determinant of agonist-induced receptor activation and signal transduction. Thus, while the Y322F mutant receptor showed normal G protein coupling, inositol phosphate signaling, and internalization, the Y322A mutant receptor has lost the ability to couple to G_q and to activate signaling responses.

Further studies were performed on the conserved DRY/S motif that is located at the junction of the third transmembrane domain and the second intracellular loop of most GPCRs. The acidic (Asp) and basic (Arg)

residues of this triplet are highly conserved throughout the GPCR superfamily. While the Arg residue in the triplet is invariant, other amino acids sometimes conservatively substitute for the Asp and Tyr residues. It has been proposed that these two residues have important functions in GPCRs, including ligand binding, receptor activation, and G protein coupling. This region of the GnRH receptor was analyzed to determine the roles of the conserved acidic residue (Asp-138), the invariant basic residue (Arg-139), and the unique Ser-140 residue (which is Tyr in most other GPCRs) in agonist-induced signal transduction and receptor internalization. The functional importance of these residues and of several polar residues in the C-terminal region of the second intracellular loop was evaluated in wild-type and mutant GnRH receptors expressed in COS-7 cells. Mutants in which Asp-138 is replaced by Asn or Glu are poorly expressed, but their internalization and inositol phosphate responses to agonist stimulation are greater than those of the wild-type receptor. In contrast, receptors in which Gln, Ala, or Ser substitute for Arg-139 show reduced internalization, and the inositol phosphate response of the Arg139Gln mutant is impaired in parallel with its low expression level. Replacing Ser-140 with Ala affects neither internalization nor signal transduction. The role of the polar amino acids in the C-terminal region of the second intracellular loop was evaluated in two additional compound mutants, Ser151Ala/Ser153Ala and Ser115Ala/Ser153Ala/Lys154Gln/Glu156Gln. Both these mutant receptors exhibit agonist-induced inositol phosphate responses similar to those of the wild-type receptor, but show increased internalization after agonist stimulation. These observations demonstrate that the conserved Asp and Arg residues in the DRY/S triplet make important contributions to the structural integrity of the receptor, and influence receptor expression, agonist-induced activation, and internalization.

Regulation of Ca^{2+} Signaling and GnRH Release by Neurotransmitters. Neurotransmitters and neuropeptides modulate the secretion of GnRH from the hypothalamus, and thus influence the release of gonadotrophic hormones into the circulation. Analysis of the biochemical and electrophysiological effects of these regulatory factors on GnRH neurons is complicated by the small number and scattered distribution of GnRH neurons in the hypothalamus, and by the consequent difficulties in isolating and identifying them. The GT1-7 line of immortalized GnRH neurons has many similarities to native GnRH neurons, and is a useful model in which to investigate the regulation and electrophysiology of the GnRH neuron. Recent evidence from GT1-7 cells suggests that the neurotransmitters glutamate and γ-aminobutyric acid (GABA), as well as the neuropeptides endothelin and GnRH, increase GnRH secretion at least in part by depolarizing the plasma membrane. This leads to increased Ca^{2+} influx through L-type calcium channels and elevated cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$), which the neuropeptides also increase by mobilizing calcium from internal stores.

The GnRH neurons of the rat hypothalamus are innervated by noradrenergic terminals, and catecholamines have been implicated in the regulation of GnRH release *in vivo* and *in vitro*. However, the role of cholinergic innervation in the control of GnRH secretion has been less extensively investigated. We observed that adding acetylcholine (ACh) to hypothalamic neurons and to GT1-7 cells elevates cytoplasmic calcium levels and induces prominent peaks in GnRH release, each followed by a sharp decline, a transient plateau, and a subsequent decline to below basal levels. Micromolar concentrations of ACh and muscarine, but not of nicotine, have a significant inhibitory action on cAMP production. The coupling of ACh receptors to G proteins is also indicated by immunoblot analyses that revealed significant reductions in both $G_{q\alpha}$ and $G_{i\alpha 3}$ after treatment of GT1-7 cells with ACh. These results suggest that ACh modulates GnRH release from normal and immortalized hypothalamic neurons by acting on specific muscarinic receptor subtypes, which activate G_q and G_i proteins that influence the activities of phospholipase C and adenylate cyclase/ion channels, with consequent effects on neurosecretion.

Regulation of Pulsatile GnRH Release by GnRH and LH Receptors Expressed in GnRH Neurons. The hypothalamic GnRH neuronal network releases GnRH in a pulsatile manner to regulate gonadotropin release from the pituitary gland, and is consequently responsible for the control of steroid hormone secretion and gamete production by the gonads. The operation of a short feedback loop between the pituitary gland and the hypothalamus is suggested by the ability of LH to modulate its own secretion *in vivo*, and to reduce single cell firing rates in the medial preoptic area and basal hypothalamus. In addition, marked inhibition of multiunit electrical activity was observed during the initiation of the preovulatory surge of LH, coincident with the late follicular rise in serum estradiol concentration. Recent studies in cultured hypothalamic neurons, as well as in immortalized pituitary gonadotrophs and in GnRH-producing neurons, have begun to address the direct involvement of neuropeptides, neurotransmitters, and pituitary hormones in the control of hypothalamic GnRH secretion. Cultured hypothalamic neurons and immortalized GnRH neurons (GT1-7 cells) exhibit pulsatile GnRH release and express plasma-membrane receptors for GnRH, prolactin, and LH, as well as for several other

hormones and transmitters. In GT1-7 cells, agonist-induced activation of GnRH receptors leads to stimulation of phospholipase C activity, increased formation of Ins(1,4,5)P_3 with mobilization of Ca^{2+} from intracellular stores and Ca^{2+} entry through voltage-sensitive calcium channels (VSCC), and modulation of GnRH release.

Treatment of GT1-7 cells with hCG caused dose-dependent stimulation of cAMP production, with a rapid increase during the first 15 minutes and a subsequent decline that can be prevented by pre-treatment with pertussis toxin. Furthermore, the stimulatory effect of cholera toxin on cAMP production can be inhibited by hCG in a dose-dependent manner. These data indicate that the LH receptors expressed in GT1-7 cells are coupled to both adenylyl cyclase stimulatory (G_s) and inhibitory (G_i) proteins. In perifused cell cultures, treatment with forskolin and 8-bromo cAMP increases the amplitude of spontaneous GnRH release. However, treatment with nanomolar concentrations of hCG abolishes pulsatile GnRH release from both GT1-7 cells and rat hypothalamic cells. The similarity of hCG action on pulsatile GnRH release to that of extracellular Ca^{2+} depletion and calcium channel antagonists, and its partial resistance to potassium-induced depolarization, suggest that it results from inhibition of plasma-membrane ion channel activity. It is probable that the inhibitory action of hCG on pulsatile GnRH release is responsible for initiating the suppression of pituitary LH secretion during pregnancy, and is mediated by a G_i-dependent mechanism that prevents neurosecretion at the hypothalamic level.

In related studies, basal cAMP production by GT-1 cells was shown to be highly Ca^{2+}-dependent and to be inhibited by the VSCC antagonist, nifedipine. Conversely, cAMP production is raised by increasing extracellular Ca^{2+}, and by the VSCC agonist BAY K8644. Increases in cAMP production induced by hCG are significantly enhanced in the presence of increased extracellular Ca^{2+}, and also by treatment with 1 nM GnRH. These findings suggest that the α subunit liberated by LH receptor coupling to G_s activates a type I calcium-dependent adenylate cyclase. The endogenous activation of LH and GnRH receptors expressed in GnRH-producing cells could promote the convergence of two distinct signaling pathways that serve to modulate GnRH production.

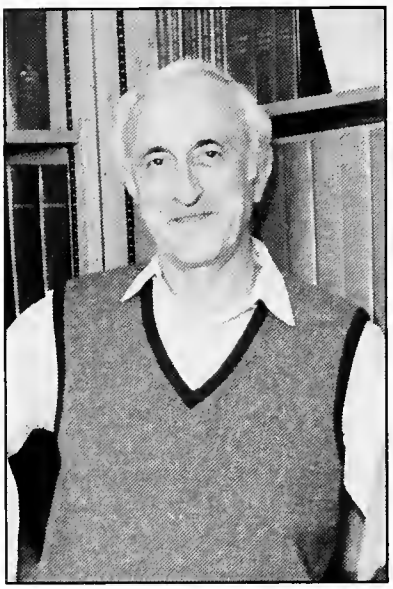
GnRH agonists also exert a biphasic effect on cAMP production by GT1 cells, with stimulation at nanomolar concentrations and inhibition at micromolar levels. However, in GnRH receptor-expressing COS cells, agonist-stimulated cAMP production exhibits a monophasic dose-response curve that is maximal at micromolar GnRH concentrations. In cultured hypothalamic neurons and GT1 cells, pretreatment with pertussis toxin (PTX) increases basal cAMP production and abolishes the inhibitory effect of high GnRH levels, but did not affect the stimulatory action of nanomolar GnRH concentrations on cAMP production. These findings are consistent with the coupling of GnRH receptors to both G_s and G_i, such that they can exert both stimulatory and inhibitory effects on adenylate cyclase signaling, depending on the prevailing agonist concentration.

Structure and Regulation of the Rat GnRH Receptor Gene. The structure of the rat gonadotropin-releasing hormone receptor gene was determined by screening a rat testis genomic library with the cDNA for the mouse GnRH receptor. The receptor gene was found to span about 20 kb of DNA and to contain three exons that encode the GnRH receptor protein. The intron-exon structure of the rat receptor gene is similar to that of the mouse and human genes, with introns located in the coding region at the sites of the fourth transmembrane domain and of the third intracellular loop. A putative TATA box is positioned 126 nt upstream of the start codon and 23 nt in front of the transcription initiation site. The 1.7 kb promoter sequence contains an SF-1 site, an AP-1 site, CCAAT sequences, and a Pit-1 binding site, and a potential CRE-like sequence. The sequences of the promoter, exons, and introns of the rat GnRH receptor gene are about 85% identical with the corresponding regions of the mouse receptor gene.

Current studies are directed at the analysis of the molecular mechanisms underlying transcriptional regulation of the receptor gene, employing luciferase receptor constructs based on selected regions of the 5′-flanking domain of the gene. For this purpose, the 1.8 kb 5′ sequence and two 5′ deleted fragments of 1.2 and 0.6 kb were subcloned into a promoterless luciferase reporter plasmid and transiently expressed in immortalized pituitary gonadotrophs (αT3-1 cells) and hypothalamic neurons (GT1-7 cells), and in COS-7 cells. Luciferase activity is significantly increased by all three fragments expressed in αT3-1 and GT1-7 cells, but not in COS-7 cells, and is highest for the 1.2 kb fragment. Treatment with forskolin or dibutyryl cAMP further increases luciferase gene expression in αT3-1 or GT1-7 cells transfected with vectors containing the 0.6 and 1.2 kb fragments. In contrast, activation of protein kinase C by phorbol 12-myristate 13-acetate has no effect on luciferase gene expression. Analysis of the sequence of the promoter region of the rat GnRH-R did not reveal a classical CRE, but a 5′-ACGCCA-3′ sequence similar to the core consensus sequence is present at position -192/-187. The use of the 27 bp oligonucleotide (-201 to -175) as a probe in gel shift assays revealed the

formation of a sequence-specific complex with nuclear proteins from αT3-1 and GT1-7 cells, but not from COS-7 cells. These findings indicate that regulatory elements for cell-specific expression are present within the 1.8-kb 5′-flanking region of the rat GnRH-R gene, and that cAMP is a candidate for controlling the expression of the GnRH-R gene.

Autocrine Actions of Endothelin in Ovarian Cancer Cells. Endothelin (ET) peptides and their receptors are expressed in numerous tissues and cell types, including the cardiovascular and nervous systems, and appear to be involved in a wide variety of physiological functions. In addition to activating phosphoinositide hydrolysis and calcium signaling, ET-1 stimulates mitogenic and proliferative responses in vascular smooth muscle and other cell types, as well as the rapid expression of c-*fos* and c-*myc*. These and other findings have demonstrated that ET-1, like several other calcium-mobilizing hormones, has the capacity to elicit growth responses in its target tissues. Recently, ET-1 has been found to be produced in a number of neoplasms, including laryngeal, lung, hepatocellular, pancreatic, colon, mammary, and cervical carcinomas. In conjunction with observations that ET-1 has TGF-β-like activity on the anchorage-independent growth of NRK 49F cells, its production by a variety of tumor cells has led to the proposal that ET-1 may participate in the pathogenesis of certain types of cancer through an autocrine/paracrine mechanism.


Kevin Catt

In recent studies, human ovarian carcinoma cells were found to produce ET-1, to express high-affinity ET_A receptors, and to exhibit cytoplasmic calcium elevations and mitogenic responses to ET-1. These responses are inhibited by the ET_A antagonist, BQ 123, which also substantially reduces the basal growth rate of unstimulated ovarian tumor cells. The findings that ET-1 is produced in ovarian cancer cells, and acts as an autocrine growth factor via ET_A receptors to stimulate calcium signaling and proliferative responses, suggest that ET-1 participates in the progression of neoplastic growth in certain ovarian tumors. The proliferative actions of ET in ovarian carcinoma cells were further investigated by the activation of mitogenic signal pathways induced by ET-1, which binds to a GPCR, and by EGF, which activates a receptor tyrosine kinase. In OVCA 433 cells, the efficacy of ET-1 as a stimulus of [^{3}H]thymidine incorporation was found to be equivalent to that of epidermal growth factor. ET-1 also stimulates the rapid expression of c-*fos*, an action that is mediated by ET_A receptors. The mitogenic action of ET-1 is not mediated by a pertussis toxin-sensitive G protein. An analysis of the effects of inhibition and depletion of protein kinase C (PKC) on mitogenic responses demonstrated that PKC is necessary but not sufficient for maximal stimulation by ET-1. In quiescent OVCA 433 cells, ET-1-induced stimulation of [^{3}H]thymidine incorporation can be prevented by two structurally distinct inhibitors of tyrosine kinase, herbimycin A and genistein. These results indicate that both PKC and protein tyrosine kinase participate in ET-1-stimulated mitogenic signaling. ET-1 rapidly stimulates tyrosine phosphorylation of several cellular proteins, of which the $pl25^{FAK}$ and p42 mitogen-activated protein kinases were identified. The mitogenic actions of ET-1 and epidermal growth factor are both potent and additive, which is consistent with the independence of their signal transduction pathways in ovarian cancer cells. These findings also indicate that intracellular signaling between the ET_A receptor and a yet unidentified tyrosine kinase is involved in the mitogenic response to ET-1.

Structure-Function Studies on AT_1 Receptors. Extensive studies on the AT_1 receptor have identified several amino acid residues and sequences that are involved in the processes of Ang II binding, agonist activation, and G protein coupling, as well as internalization of agonist-receptor complexes. However, more information is needed about the individual residues involved in these processes, and the intramolecular interactions between specific regions of the receptor that lead to the conformational changes necessary to adopt the active conformation of the receptor. This problem applies not only to the Ang II receptors, but also to all members of the G protein-coupled (GPCR) superfamily. The binding site for the non-peptide antagonist, losartan, was previously shown to be located within the transmembrane domains of the receptor. We have also identified residues within the cytoplasmic tail of the receptor that are essential for agonist-induced internalization of the ligand-receptor complex. Current studies are focused on the identification of residues that are involved in the agonist-induced activation of the receptor and in its coupling to the plasma-membrane effector systems that

mediate intracellular signaling responses. Two of these amino acids have been identified as Tyr-215 in the fifth transmembrane domain, and a conserved apolar residue (Leu-222) in the subjacent region of the third cytoplasmic loop of the AT_1 receptor.

Although all GPCRs share a seven-transmembrane structure similar to that of rhodopsin, relatively few amino acids are highly conserved among the super-family of G protein-coupled receptors. In most GPCRs, the binding site for physiological agonists is formed by amino acids located in superficial regions of the intramembrane helices and of the extracellular regions of the receptor. The conformational change evoked by agonist binding is transmitted by the transmembrane helices to the intracellular loops, which couple the receptor to its cognate G protein(s) and thence to intracellular signaling systems. In particular, the amino and carboxyl-terminal regions of the third intracellular loop are known to be involved in receptor activation and G protein recognition. The former region appears to be an amphophilic α-helical extension of the fifth transmembrane helix, and synthetic peptides corresponding to its sequence have been shown to activate the appropriate G proteins. However, relatively few of the amino acids that are required for receptor activation have been identified, and the structural determinants of the G protein coupling specificities of GPCRs have not been defined. Identification of other amino acids that determine G protein coupling is important in providing clues to the nature of the interaction between the receptor and its G proteins(s) and of the mechanisms of G protein activation and specificity.

Previous deletion studies and a recent report utilizing chimeric AT_1/AT_2 receptors have identified the amino-terminal portion of the third intracellular loop as a critical region for G protein coupling of the rat AT_{1a} receptor. We recently observed that substitution of phenylalanine for a conserved tyrosine residue (Tyr-215) in the fifth transmembrane domain, near the origin of the third cytoplasmic loop, causes loss of AT_{1a} receptor activation. Analysis of the amino acids in this region of the AT_1 receptor identified Leu-222 as another essential residue in receptor activation by Ang II. Replacement of Leu222 with polar or charged residues causes marked impairment of inositol phosphate signaling and of receptor internalization, without affecting receptor affinity for the peptide antagonist, [Sar^1,Ile^8]Ang II. Although such mutants show normal antagonist binding, their affinity for Ang II is markedly reduced, indicating that the mutant receptor is unable to adopt the high affinity conformation. Since many G_q-coupled receptors contain an apolar amino acid (frequently leucine) in the position corresponding to Leu-222 of the AT_{1a} receptor, it is probable that this conserved residue in the third intracellular loop is a key element in the agonist-induced activation of many GPCRs.

Roles of Seventh Transmembrane Domain Amino Acids in AT_1 Receptor Function. The seventh transmembrane domain appears to be an important region both for ligand binding and for activation of the AT_1 receptor and several other GPCRs. Mutations of amino acid residues in the seventh helix have ben reported to interfere with the binding to non-peptide antagonists. These residues include: Tyr-292, the amino acid located in the position at which the retinal chromophobe binds covalently to the rhodopsin molecule; Leu-300 and Phe-301, two apolar amino acids located in the center of the conserved NPXXY sequence; and two polar residues, Asn-294 and Asn-295, located between Tyr-292 and the NPXXY sequence. Furthermore, mutations in the seventh transmembrane helix have been found to interfere with the binding to peptide ligands and with signal transduction from the receptor. Recent reports have suggested that an interaction of Asn-295 or Tyr-292 with Asn-111 in the third helix stabilizes the inactive conformation of the receptor, based on the finding that mutation of Asn-295 or Asn-111 causes constitutive activation of the AT_1 receptor. Since structure-function studies suggest that the seventh transmembrane domain is a region in which the binding of AT_1 peptide and non-peptide ligands overlaps, the role of this region in the binding of non-peptide agonists was analyzed. Receptors bearing alanine replacements of two adjacent polar residues, Asn-294 (N294A) and Asn-295 (N295A), were expressed in COS-7 cells to study the importance of this region in the ligand binding and activation functions of the AT_{1a} receptor.

The N294A receptor showed a substantial reduction in binding affinity for all Ang II ligands tested, including both peptide and non-peptide agonists, with no change in the maximal inositol phosphate response and internalization rate of the mutant receptor. Replacement of Asn-295 with a serine residue impairs non-peptide antagonist binding to the receptor, but has only a minor effect on its Ang II binding affinity. This contrasts with the impaired ability of the N295A mutant to bind to peptide ligands. It is possible that the hydroxyl group of the introduced serine residue can better substitute for the side-chain of the asparagine residue when Ang II binds to the receptor. Consistent with this notion, the amphibian angiotensin receptor and the mammalian AT_2 receptor, which both have serine residues in the position corresponding to Asn-295 of the mammalian AT_1 receptor, exhibit high affinity for Ang II but very low affinity for AT_1-selective non-peptide antagonists.

The N295A mutation also had a deleterious effect on the binding of non-peptide agonist ligands, in contrast to previous studies in which replacement of Asn-295 by aspartic acid (N295D) was found to have no effect on Ang II and non-peptide agonist binding. However, the efficacy with which L-162,313 and other non-peptide agonists stimulate inositol phosphate responses is greatly reduced in the N295D mutant. The reduced affinity of the N295A receptor for Ang II and its non-peptide agonists appears to be distinct from the effect on antagonist binding, since it is only observed after alanine substitution. These findings suggest that the interaction of Asn-295 with agonist ligands (e.g., by hydrogen bonding) might provide energy for their binding to the receptor, and could account for the role of Asn-295 in non-peptide agonist-induced inositol phosphate responses. The marked effect of the N295A mutation on non-peptide agonist affinity underlines the importance of the intramembrane pocket in agonist binding to the AT_1 receptor, and supports the conserved role of intramembrane helices in the agonist-induced conformational change during receptor activation.

Mutation of Asn-295 to serine has been reported to cause constitutive activation of the AT_1 receptor, possibly reflecting an interaction between Asn-295 and Asn-111 (in the third transmembrane helix) that normally stabilizes the inactive conformation of the AT_1 receptor. However, constitutive activation was not observed with N295D, another replacement of this residue, and was not detectable in the N295A receptor. Thus, it is possible that the constitutive activity of the N295S mutant might be due to an interaction of the serine residue, rather than loss of the asparagine residue, as originally suggested. While the Asn-295 residue is clearly important in ligand binding, the adjacent Asn-294 residue was found to have a major role in receptor activation. Although the N294A receptor has markedly reduced affinity for losartan, its binding affinity for [Sar^1,Ile^8]Ang II is completely normal and its affinities for peptide and non-peptide agonists are moderately but consistently reduced. The most prominent feature of the N294A receptor is its attenuated ability to engender inositol phosphate responses even in the presence of high concentrations of Ang II. The moderately impaired agonist binding of the N294A receptor may reflect a loss in its ability to achieve the conformation that is required for agonist-induced activation.

Cloning of Phosphatidylinositol 4-Kinase(s) Essential for Agonist-Induced Ca^{2+} Signaling. We recently identified a novel wortmannin-sensitive PI 4-kinase that is required for the biosynthesis of precursor phosphoinositide pools that are utilized for the $InsP_3/Ca^{2+}$ signaling pathway in agonist-stimulated cells. This finding was based on the observation that the fungal metabolite, wortmannin, a potent inhibitor of myosin light chain kinase, prevents the sustained but not the initial increases in $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ in a variety of agonist-stimulated cells, including adrenal glomerulosa cells, NIH 3T3 fibroblasts, and Jurkat lymphoblasts. The inhibition of Ca^{2+} influx by wortmannin was found to be a consequence of reduced $Ins(1,4,5)P_3$ formation and probably reflects the resultant refilling of the agonist-sensitive Ca^{2+} pool. Micromolar concentrations of wortmannin were found to inhibit the synthesis of hormone-sensitive PtdIns(4)P and $PtdIns(4,5)P_2$ pools in intact adrenal glomerulosa cells, and a wortmannin-sensitive PtdIns 4-kinase was identified in adrenocortical extracts. Inhibition of this PtdIns 4-kinase by wortmannin results in rapid loss of the hormone-sensitive $PtdIns(4,5)P_2$ pool in angiotensin II-stimulated glomerulosa cells.

Although receptor-regulated formation of the calcium-mobilizing second messenger, $Ins(1,4,5)P_3$, is highly dependent on the formation of $PtdIns(4,5)P_2$ by PtdIns 4-kinase and PtdIns(4)P 5-kinase, the PtdIns 4-kinase involved in this pathway has not yet been identified. Most of the cellular PtdIns 4-kinase activity is located in the membrane fraction, and can be solubilized by detergents. The brain contains two forms of detergent-soluble PtdIns 4-kinase: type II, a smaller (about 56 kDa) adenosine-sensitive enzyme; and type III, a larger (over 200 kDa) form that is less sensitive to inhibition by adenosine. The wortmannin-sensitive cytosolic PtdIns 4-kinase that we have identified in the bovine adrenal cortex, and which is essential for the maintenance of agonist-sensitive $PtdIns(4,5)P_2$ pools in several cell types, is a type III PtdIns 4-kinase that is distinct from the tightly membrane-bound, Ca^{2+}- and adenosine-sensitive, type II PtdIns 4-kinase. The type III PtdIns 4-kinase prepared from bovine brain exhibits kinetic and other parameters that are similar to those of the soluble adrenal enzyme. These findings indicate that type III PtdIns 4-kinases, rather than type II, are responsible for the maintenance of the precursor phospholipids required for intracellular signaling through the inositol phosphate/Ca^{2+} pathway. In contrast, the more abundant type II PtdIns 4-kinases are not wortmannin-sensitive, and do not appear to participate in the synthesis of hormone-sensitive phosphoinositide pools.

The wortmannin-sensitive type III PI 4-kinase, which is loosely membrane-associated, was purified from the bovine adrenal cortex as a mixture of two enzyme activities with molecular sizes of about 200 and 110 kDa. [^{3}H]wortmannin labeling and subsequent SDS-PAGE analysis confirmed the existence of two separate proteins of 210 kDa and 110 kDa, both of which show catalytic properties characteristic of type III PI 4-kinases and

similar wortmannin sensitivities. Peptide sequences obtained from the 210-kDa enzyme correspond to those of a recently described rat 230-kDa PI 4-kinase. A 6.5-kb cDNA containing an open reading frame of 6129 nucleotides, which encodes a 230-kDa protein, was isolated from brain cDNA. Northern blot analysis of human mRNA revealed a major 7.5-kb transcript. Based on peptide sequences from the smaller enzyme, a 3.9-kb cDNA with an open reading frame encoding a 90-kDa protein was isolated from a bovine brain cDNA library. Expression of this cDNA in COS-7 cells yields a 110-kDa protein with wortmannin-sensitive PI 4-kinase activity. Northern blot analysis of a human mRNA panel showed a single transcript of about 3.8 kb. The molecular cloning of these novel wortmannin-sensitive type III PI 4-kinases will allow detailed analysis of their signaling and other regulatory functions in mammalian cells.

Ang II-Induced Signaling and Growth Responses in Glomerulosa Cells. Ang II controls the growth and differentiation of the *zona glomerulosa* and stimulates aldosterone production, whereas ACTH controls glucocorticoid secretion by the *zona fasciculata* and also acts in the *zona glomerulosa* as a potent stimulus of aldosterone secretion. In several Ang II target tissues, AT_1 receptors interact with two distinct guanine nucleotide binding proteins, $G_{q/11}$ and G_i, to activate phospholipase C and to inhibit adenylate cyclase, respectively. We previously demonstrated that Ang II increases [^{3}H]thymidine incorporation in primary cultures of bovine adrenal glomerulosa cells after first five days of culture. At this time, Ang II increases the proportion of cells in S phase and does not cause accumulation of cells in the G_2 phase. Ang II also stimulates the proliferation of cultured glomerulosa cells during prolonged treatment, an effect mediated by AT_1 receptors. These results indicate that intracellular mechanisms that mediate growth responses become more active during sustained culture of glomerulosa cells, and that Ang II exerts potent mitogenic actions that depend on the functional state of the glomerulosa cells.

Although Ang II is a well-recognized stimulus of growth and mitogenesis in bovine adrenal glomerulosa cells, little is known about the signaling pathways that mediate these responses. An analysis of the growth-promoting pathways in cultured glomerulosa cells revealed that Ang II causes rapid but transient activation of mitogen-activated protein kinase (MAPK), with an ED_{50} of 10-50 pM. This effect is mediated by the Ca^{2+}-mobilizing AT_1 receptor, and extracellular Ca^{2+} is required for an optimal response. However, release of Ca^{2+} from intracellular stores alone is insufficient to activate MAPK. Ang II-induced activation of MAPK is only slightly affected by pertussis toxin pretreatment, but is partially inhibited by micromolar concentrations of wortmannin, suggesting a possible role for phosphatidylinositol kinases in the response. Although activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate is sufficient to activate MAPK, PKC depletion only partially attenuates Ang II-induced MAPK activation. Ang II also induces the rapid activation of ras and raf-1 kinase, both of which correlate temporally with activation of MAPK. In addition, we found that raf-1 exhibits a later mobility shift, reflecting hyperphosphorylation of the enzyme, that correlates with deactivation of the kinase. These findings indicate that Ang II acts through AT_1 receptors to stimulate multiple pathways to MAPK activation operating via both PKC and ras/raf-1 cascades in bovine adrenal glomerulosa cells.

Expression of Angiotensin AT_1 Receptor Sequences. Methods have been developed for production of peptide sequences and entire proteins corresponding to the AT_1 and GnRH receptors. These products have been used to prepare improved anti-receptor antibodies, and will be applied to study the properties of receptor proteins, which will include a structural analysis. In addition to expression of the C-terminal and N-terminal fragments of the rat AT_{1b} receptor fused to maltose binding protein in bacterial cells, the cDNA encoding the full length AT_{1b} receptor (1.08 kb) was cloned into selected *Baculovirus* transfer vectors containing the glutathione-S-transferase (GST) reading frame and expressed in Sf9 cells. Although cells infected with similar vectors without GST displayed high Ang II binding activity, transfected cells expressing the GST-tagged receptor did not, probably due to hindrance by the N-terminal GST moiety. Purification of the solubilized GST fusion proteins by glutathione affinity chromatography gave yields in the range of 60-70%, with immunoreactive receptor components corresponding to 60-80 kDa, 110 kDa, and 210 kDa proteins. Cleavage of the fusion proteins with human thrombin yielded two major components of 50 kDa and 28 kDa, and microsequencing of the 50 kDa band gave (G0S)-M-T-L-X-S-S-T-Q-D-G-I-K-R-I-Q-E. This is consistent with the N-terminal sequence of the $AT1_b$ receptor, except that the dipeptide sequence in parentheses represents an added thrombin cleavage site and X is a blank rather than N as encoded by the cDNA sequence constructed for expression. The blank site is also a consensus glycosylation site (N*SS). Since the insect *Sf9* cell glycosylates at the same site as mammalian cells, it is likely that this N is glycosylated by post-translational modification. The 28 kDa band has the amino acid

sequence of GST. Thus, the GST fusion protein of the AT_{1b} receptor was purified in good yield, and the thrombin cleavage product of the fusion protein permitted the identification of a glycosylation site at N*SS.

INTRACELLULAR SIGNALING IN ENDOCRINE CELLS

Anterior pituitary gonadotrophs and the hypothalamic neurons that regulate their functions are excellent cell models for studies on cellular calcium homeostasis. ***Stanko Stojilkovic*** and his colleagues in the Unit on Cellular Signaling have utilized these cells to elucidate the mechanisms underlying plasma membrane-derived and endoplasmic reticulum-derived calcium oscillations and calcium-controlled cellular functions. The current emphasis of these investigations is on the role of voltage-gated Ca^{2+} influx in the refilling of intracellular Ca^{2+} stores, and the control of enzyme activity and neuropeptide/hormone secretion. In addition, the role of Ca^{2+} signals in controlling plasma membrane-associated potassium channels and endoplasmic reticulum (ER)-associated inositol (1,4,5)-trisphosphate ($InsP_3$) gated-channels is being addressed.

Sensing and Refilling Calcium Stores in Pituitary Gonadotrophs. In excitable and non-excitable cells, elevations in cytosolic calcium concentration ($[Ca^{2+}]_i$) occur via receptor-controlled Ca^{2+} release from the ER, and are commonly expressed as prominent $[Ca^{2+}]_i$ oscillations. Such agonist-induced Ca^{2+} mobilization reduces the size of the intracellular Ca^{2+} stores, leading to increased Ca^{2+} influx via the mechanism known as capacitative Ca^{2+} entry. In a number of nonexcitable cell types, capacitative entry is believed to occur through a Ca^{2+} release-activated Ca^{2+} channel. Several hypotheses have been proposed to explain how the store content regulates these channels, ranging from the existence of diffusible cytoplasmic messengers to interactions between proteins on the ER and plasma membrane. In excitable cells, Ca^{2+} entry through voltage-sensitive Ca^{2+} channels is essential for refilling internal Ca^{2+} stores. However, the manner in which the store content controls influx through these channels is not clear. To address this question, pituitary gonadotrophs were employed as a cell model for experimental and theoretical studies. Modeling was done in collaboration with Yue-Xian Li and John Rinzel at the Mathematical Research Branch of the NIDDK, and Joel Keizer of the Institute of Theoretical Dynamics, University of California at Davis. The store content in unstimulated and agonist-stimulated cells, the time-course and concentration dependence of gonadotropin-releasing hormone (GnRH)-induced depletion of the agonist-sensitive Ca^{2+} pool, as well as dynamic changes in $[Ca^{2+}]_i$ and membrane potential prior to, during, and after GnRH pulses were measured and compared with theoretical predictions. Results from these investigations indicate that cytosolic Ca^{2+} in the vicinity of the plasma membrane acts as a messenger for ER, connecting via Ca^{2+}-activated K^+ channels and Ca^{2+} pumps in the plasma membrane. Thus, in excitable cells that do not express store-operated Ca^{2+} channels, cytosolic Ca^{2+} profiles provide a sensitive mechanism for regulating net Ca^{2+} flux through the plasma membrane during store depletion and refilling.

Role of Voltage-Gated Ca^{2+} Entry in Phospholipase D Activation. It is well established that the excitability of GnRH neurons is essential for episodic neuropeptide release, but the mechanism by which electrical activity controls GnRH secretion has not been elucidated. In collaboration with Kevin Catt and coworkers in the Section on Hormonal Regulation, the role of phospholipase D in mediating the electrical activity-dependent neuropeptide secretion was investigated in immortalized GT1 neurons. These cells secrete GnRH and express GnRH receptors, activation of which is associated with a transient hyperpolarization of GT1 cells, followed by sustained firing of action potentials. This is accompanied by an increase in phospholipase D activity, as indicated by elevated phosphatidylethanol (PEt) production. GnRH-induced PEt production is reduced by inhibition of phospholipase C-dependent phosphoinositide hydrolysis by U73122 and neomycin, suggesting that signaling from phospholipase C leads to activation of phospholipase D. The intermediate role of protein kinase C in this process was indicated by the ability of the phorbol ester PMA to induce time- and dose-dependent increases in PEt and in diacylglycerol, but not in $InsP_3$, and by reduction of GnRH-induced PEt accumulation in protein kinase C-depleted cells. Consistent with the role of action potential-driven calcium entry in this process, agonist-induced phospholipase D activity was also found to be reduced by nifedipine and by removal of extracellular Ca^{2+}. Inhibition of the phospholipase D pathway by ethanol and propranolol reduces diacylglycerol production and causes a concomitant reduction in GnRH release. These data indicate that voltage-gated Ca^{2+} entry and protein kinase C act in an independent but cooperative manner to regulate phospholipase D activity, which contributes to the secretory response in GT1 cells. Thus, the electrical activity of GnRH-secreting neurons participates in the functional coupling between GnRH receptors and phospholipase D pathways.

Wortmannin-Sensitive and -Insensitive Steps in Ca^{2+}-Controlled Exocytosis. In many secretory cells, Ca^{2+} appears to be the major, if not exclusive, messenger that is responsible for regulated exocytosis. Several hypotheses have been proposed to explain the mechanism of Ca^{2+}-induced secretion. In one proposal, it has been suggested that myosin light chain kinases (MLCKs) play a role. MLCKs belong to a heterogeneous group of enzymes that are broadly classified into invertebrate striated muscle and vertebrate smooth muscle/nonmuscle types. In cultured rat pituitary cells, the role of MLCKs was investigated in depolarized and agonist-stimulated cells, both of which increase $[Ca^{2+}]_i$ and LH release. Treatment of pituitary cells with the MLCK inhibitor, wortmannin, was found to attenuate GnRH-induced LH release. Wortmannin also reduces the LH responses to non-receptor mediated elevation of $[Ca^{2+}]_i$ by ionomycin and activation of voltage-gated Ca^{2+} influx by Bay K 8644 or high extracellular potassium concentration, as well as Ca^{2+}-induced LH release in permeabilized pituitary cells. The $[Ca^{2+}]_i$ responses to these stimuli are unaltered in wortmannin-treated pituitary cells, indicating that this compound inhibits a Ca^{2+}-dependent step in exocytosis without affecting Ca^{2+} signaling. In perifused pituitary cells, the GnRH-induced early spike phase of LH release is not affected by wortmannin, whereas the subsequent plateau phase is almost completely inhibited. No significant changes in GnRH-induced phospholipase D activity and diacylglycerol production were observed in wortmannin-treated pituitary cells during the sustained phase of agonist stimulation. Wortmannin also has no effect on LH responses to the protein kinase C activator, PMA, further indicating that the attenuation of agonist-induced LH release is not related to inhibition of the diacylglycerol/protein kinase C pathway. In addition, agonist-induced LH release is attenuated by two other MLCK inhibitors, MS-347a and KT5926. These data suggest that MLCK mediates the downstream effects of Ca^{2+} on exocytosis, an action supported by the finding of wortmannin-sensitive phosphorylation of a 20-kDa protein in pituitary cells and in αT3-1 gonadotrophs treated with GnRH, potassium, and Bay K 8644. This protein co-precipitates from pituitary extracts with a specific antibody to non-muscle myosin IIB and co-migrates with 20-kDa smooth muscle myosin light chain on SDS-PAGE. These results demonstrate that Ca^{2+} controls exocytosis through an initial wortmannin-insensitive step and a sustained wortmannin-sensitive step, and suggest that the latter event in the cascade of cellular responses is dependent on phosphorylation of non-muscle myosin IIB light chain by MLCK.

Bidirectional Effects of Voltage-Gated Ca^{2+} Entry on $InsP_3$-Induced Calcium Release. Calcium itself acts as a coagonist with $InsP_3$ in controlling periodic Ca^{2+} release from the ER. Both facilitatory and inhibitory effects of calcium on $InsP_3$-induced Ca^{2+} release have been indicated. It has also been suggested that calcium-dependent inactivation of $InsP_3$-induced Ca^{2+} release is a slower process than activation; it has been suggested that inactivation occurs in a sub-second to second time scale, whereas activation occurs in a low msec time scale. To study the role of Ca^{2+} on $InsP_3$-induced Ca^{2+} release in gonadotrophs, we explored the influence of voltage-gated Ca^{2+} entry on the kinetics of $[Ca^{2+}]_i$ oscillations triggered by GnRH and by injection of $InsP_3$ into the cytoplasm. Under physiological conditions, the frequency of the GnRH-induced oscillations increases with time, while the amplitude falls until both reach stable values. However, in hyperpolarized cells (below -50 mV) both parameters progressively fall until the signal is abolished, and these effects are reversed by depolarization of the membrane positive to -45 mV. Depolarization also leads to an increase in the periods during which $[Ca^{2+}]_i$ remains elevated, due to an increase in spike duration and a decrease in the interspike interval. The depolarization-driven recovery of the frequency is instantaneous, whereas recovery of the amplitude of Ca^{2+} spiking is gradual. Several lines of evidence indicate that activation of L-type Ca^{2+} channels leads to the modulation of $[Ca^{2+}]_i$ profiles. In particular, this mechanism facilitates Ca^{2+} liberation at lower $[Ca^{2+}]_i$ levels due to its direct and instantaneous action on release mechanisms, and magnifies the Ca^{2+} signal due to its gradual effect on the balance between the efflux and influx of Ca^{2+}.


Stanko Stojilkovic

Identification of a Novel Ca^{2+}-Dependent Current in Pituitary Gonadotrophs. In general, the coupling factors between voltage-dependent Ca^{2+} entry and $InsP_3$-dependent Ca^{2+} release are calcium-sensitive currents. In gonadotrophs, apamin-sensitive Ca^{2+}-activated potassium channels are the predominant mediator of the Ca^{2+}-dependent plasma membrane conductance. The apamin-sensitive channels are also expressed in other

pituitary cells types, including corticotrophs, lactotrophs, thyrotrophs, and immortalized GH_3 cells, as well as in other endocrine cells. In cultured rat pituitary gonadotrophs, GnRH-induced oscillations in $[Ca^{2+}]_i$ are associated with periodic membrane hyperpolarization. The hyperpolarizing waves are secondary to the activation of apamin-sensitive Ca^{2+}-activated potassium channels that account for a single class of ^{125}I-apamin binding sites present in these cells. In a substantial fraction of gonadotrophs, however, a Ca^{2+}-controlled oscillatory current that is resistant to apamin was observed, even at inhibitor concentrations five orders of magnitude higher than the K_d observed in binding experiments. With K^+ in the pipette, the apamin-resistant current shows a reversal potential of -42 mV, nearly 40 mV more depolarized than that of the apamin-sensitive current. With Cs^+ in place of K^+, both the size of the apamin-insensitive current and its reversal potential remain unchanged. Ion substitution studies further revealed that the reversal potential is independent of Cl^-. In contrast, a 11 mV hyperpolarizing shift in the reversal potential occurs when extracellular Na^+ is reduced to 80 mM. In cells expressing apamin-resistant conductances, addition of apamin evokes a marked increase in the duration of the action potentials and a reduction in the frequency of spontaneous action potential spiking. In the presence of GnRH, gonadotrophs exhibit the typical bursting pattern of electrical activity. Further exposure of the cells to apamin depolarizes the membrane from a silent phase bursting level of about -80 mV to a new level of about -40 mV. These observations indicate that, in addition to apamin-sensitive current, a subpopulation of pituitary gonadotrophs also express cationic-selective Ca^{2+}-activated membrane conductances that have the potential to remodulate spontaneous and agonist-induced electrical activity.

Tamas Balla

MOLECULAR BASIS OF CELL REGULATION BY LIPID-DERIVED MESSENGERS

The Unit of Molecular Signal Transduction, led by ***Tamas Balla***, was established in 1997 and will investigate the role of phospholipid messengers in mediating the actions of hormones and growth factors. Research will be focused on the mechanisms by which phosphatidylinositol kinases regulate cellular functions and on the relationship between these events and calcium signaling. Definition of the molecular basis of protein-lipid interactions and their importance in the activation of cellular responses by G protein-coupled receptors and receptor tyrosine kinases will also be subjects of the Unit's investigations.

HORMONAL CONTROL OF GONADAL FUNCTION

The Section on Molecular Endocrinology, under the direction of ***Maria Dufau***, investigates the molecular basis of peptide hormone action, with particular emphasis on the control of gonadal function. This includes analysis of the structure and function of gonadotropin receptors, as well as the hormone-regulated membrane coupling and intracellular events involved in the modulation of steroid biosynthesis in the testis and ovary. Current research also includes studies on cell-to-cell communication in the testis, developmental aspects of Leydig cell maturation, and induction of regulatory mechanisms in the Leydig cell. This group also investigates the properties and biological activity of stored and circulating gonadotropins in physiologic regulation and in clinical disorders of pituitary and gonadal function.

Role of Leucine-Rich Domains in Exons 1-4 of the LH/hCG Receptor for Hormone Binding Activity. The luteinizing hormone receptor (LHR) belongs to a G protein subclass that includes receptors for pituitary and placental glycoprotein hormones LH, FSH, and hCG. The LHR is composed of two functional units, the extracellular hormone binding domain and the seven-transmembrane cytoplasmic module. The hormone binding region was localized to the N-terminal (1-7) exons of the extracellular domain where four Cys residues in exon 1 are essential for binding to hormone, and two other Cys (exons 5 and 6) lie within the leucine repeat pocket structure (exons 1-8). This region also contains a leucine zipper domain. The LRR domains of the glycoprotein hormone receptors show about 40% sequence similarity. The LRR (LXXLXL) sequence is in the beta

sheet that flanks protein contact points in the crystal structure of the ribonuclease inhibitor, while the leucine zipper motif (X6L) is an α-helix, which could serve as a protein binding domain.

Studies were conducted to elucidate the function of these hydrophobic receptor domains in hormone-receptor interaction and specificity. For this purpose, insertion and point mutations of the LHR were targeted at amino acids that, if mutated in the FSHR would create, abolish, or insert a leucine zipper. Mutant LHRs were expressed in mammalian COS1 and in insect SF9 cells, and gonadotropin binding activity, specificity, and expression of mutant LHRs were determined by hormone binding assay and specific radioimmunoassay. Substitution of Leu-39 for Val-39 (V39L) in exon 2, which introduces a leucine zipper in exons 1 and 2 of the LHR, abolishes hCG binding. This loss can be recovered by the conservative amino acid substitution of Val-39 to Ile-39 (V39I). Insertion of the unique exon 1 nine amino acids of the FSHR (TDLPRNAIE, called FSHRI) which extends the artificial leucine zipper in exons 1 and 2 of the LHR, does not restore hCG binding activity nor does it confer FSH binding. A Leu to Arg mutation at position 82 of the LHR (L82R), which results in the loss of the leucine-zipper motif in exons 3 and 4, causes a 70% reduction of hCG binding activity, with preservation of binding affinity. Since the expression of this receptor in the cell membrane is equivalent to that of the wild-type, one can conclude that the mutated receptor is not trapped intracellularly. Also, no molecular weight differences were detected by Western blot analyses, and the reduction in binding activity is attributable to changes in the conformational stability of the altered receptor. Mutation of Leu-29 of the first LRR domain in exons 1and 2 to Glu completely abolishes hormone binding, indicating that this leucine and/or LRR is essential for the formation of the receptor binding site. The abolition of hormone binding by insertion (V39L and FSHRI) or deletion (L82R) of leucine zippers in exons 1-4 probably results from perturbation of structural hormone interaction sites, or the specific deletion of a hormone contact point on the receptor. Since none of these changes confer FSH binding capacity, additional regions are either required for the binding reaction or participate in the binding site.

The Human LH Receptor Gene: Evidence for Multiple Promoter Domains and Gene Diversity. We have previously performed extensive studies on the rat LHR gene and its transcriptional regulation. The promoter for the rat TATAless/Inr gene has been has been localized to the -174 bp 5′ flanking region. SP1-mediated transcriptional activation was demonstrated, and domains upstream of -174 inhibit basal transcriptional activity. A mechanism of regulation was proposed involving competition for overlapping AP-2/NF1 domains within the promoter region and an upstream inhibitory R domain. Studies on the promoter of the rat LHR gene indicate that the 1882 bp domain upstream of the 174 bp promoter region markedly inhibits basal promoter activity. We next proceeded to characterize the transcriptional regulation of the human LH receptor.


Maria Dufau

Unexpectedly, the human LHR gene that we isolated from a placental library (Gene II) differs from that previously isolated from a lymphocyte library by others (Gene I) by several base changes in the 5′ flanking region, and has a deletion of six nucleotides in the coding region (+55 to +60) that conforms to the sequence of the human ovarian cDNA. The human LHR gene also differs in the predicted (not demonstrated) location of the promoter domain. Restriction maps demonstrated that both coding regions are present in the placental genomic library. Gene dosing revealed four copies of the human LHR in chromosome 2p16-21, in contrast to a single copy in the rat. Gene II promoter activity was localized exclusively to the 176 bp domain 5′ to the initiation codon, and multiple initiation sites (TSS) were identified within this domain in the human testis, ovary, and in JAR (human choriocarcinoma) cells. In addition, the testis and JAR cells exhibit transcriptional start sites 5′ of -176, although no promoter activity was apparent upstream of the -173 domain. Significant levels of promoter activity in JAR cells can be attributed to the absence of upstream inhibitory domains that are present in the rat LHR gene. Two Sp1 domains at -79 and -120 bp were found to be of major importance for transcriptional activity, and inhibitory activity was identified at an AP2 element (-67 bp) and an ERE half site (-171). Mutation of the AP2 element at 67 bp results in 80% stimulation of transcriptional activity. Gel retardation studies indicate that the inhibitory AP2-1 element located immediately 3′ of the major TSS at -70 bp, which is similar to the non-consensus activator Sp1-4 element in the rat, binds to a protein that does not react with an AP2

antiserum but is supershifted by Sp1 antibody. The inhibition of transcription by AP2 is independent of Sp1-1-induced or Sp1-2-induced activation. It is likely that this element does not bind to an activating Sp1 protein, but rather to a transcription factor that is immunologically related to SP1. The location of the inhibitory AP2 element 3′ of the functional Sp1 domain suggests that steric blocking of preinitiation complex/polymerase activity by the AP2 binding *trans*-factor may contribute to its mode of inhibition.

The presence in the human gene of an ERE nuclear element half-site, with a degenerate second half-site that is an imperfect version of the GRE, suggests that members of the nuclear receptor family regulate the LHR promoter. In the JAR cell, mutation of only the ERE half-site increases promoter activity by 100%. Gel retardation of this domain in the presence of JAR nuclear extract revealed three protein/DNA species. Nuclear extract competition for the three species is abolished upon mutation of the ERE alone, or both the ERE and GRE. However, mutation of the GRE does not abolish competition for any of the three protein/DNA species. None of three hER antibodies that recognize epitopes in the N-terminal activation domain or steroid binding domain, nor antisera to the orphan nuclear receptors hERα1 and SF1, supershift in the protein/LHR ERE/GRE complex. Furthermore, estradiol does not affect promoter activity. These studies indicate that the estrogen receptor is probably not a factor in p176 promoter regulation. Thus, it is possible that the transcriptional factors that bind to this domain in the transfected cells are members of the orphan or ligand-independent family.

Other studies were carried out to evaluate hormone-induced changes in promoter activity (p176GL) by mediators of receptor-induced activation. In this case, cAMP was used instead of the hormone because the LH receptors expressed in the human JAR cell line do not bind to hormone, presumably due to a deletion at exon 2/3 and 3/4 junctions. Addition of cAMP to JAR cells transfected with the p176GL construct increases promoter activity up to three-fold. Furthermore, cAMP activation does not appear to be mediated through removal of the inhibitory transfactors at the AP2 or ERE domains. Regulation of the LHR gene II 176 bp promoter transcription appears to be based on the independent contributions of basal inhibitory activity exerted through the AP-2 and ERE elements, and of cAMP-mediated activation. The ability of cell-surface LH receptors to bind to hormone and to generate cAMP may be a major determinant in controlling activation or inhibition of the p176 promoter, where positive and negative feedback loops are predicted to participate in the regulated expression of the specific LHR gene II.

The Prolactin Receptor Gene: Steroidogenic Factor-1 Dependence of Prolactin Receptor Promoter I. Previous studies from our laboratory have provided insights into the complex control of PRLR gene expression by demonstrating multiple tissue-specific promoters of the rat prolactin receptor gene. The expression of the prolactin receptor is under the control of two tissue-specific promoters (PI, gonads; PII liver) and one common (PIII) promoter. The latter was recently found to be the sole promoter utilized in the mammary gland. The order of the three promoters in the gene was determined by mapping genomic clones: the relative orientation was found to be 5′-PIII-PI-PII-3′. In further studies, we have investigated the underlying mechanisms of the tissue-specific control in the gonads, specifically of the $E1_1$ non-coding exon by promoter I. Deletion analyses identified a 152 bp region (-700 to -549) as the minimal promoter domain of the PRLR gene, and revealed that sequences upstream of -700 do not influence the transcriptional activation of this promoter. Although sequence analyses of the 5′-flanking region did not show a consensus TATA-box, two adjacent TATA-like sequences were located 10 and 23 bp 5′ of the TSS.

The PI promoter belongs to the class of TATA-less/non-initiator, GC-box-less gene promoters. This promoter contains a steroidogenic factor 1 (SF-1) element, CCAAGGTCA (-676/-668), positioned 119 bp upstream of the transcription initiation site, which binds to the SF-1 protein of nuclear extracts from gonadal cells. Mutation of the SF-1 binding site abolishes the SF-1 binding activity and markedly reduces promoter activity to close to basal levels (by 8 to 10-fold) in MLTC cultures as well as in primary cultures of granulosa and Leydig cells. Mutation of the CCAAT box (-629/623) caused a small but significant reduction (10%) in promoter activity in the absence of the SF-1 sequence. In contrast, the two adjacent TATA-like sequences exert marked inhibition of promoter I, and their mutation caused a doubling of basal promoter activity. Although recent studies have demonstrated that cAMP significantly increases basal promoter activity, this effect is independent of SF-1. Our studies have demonstrated that the SF-1 protein binds to its cognate regulatory element within the PI promoter domain and exerts a dominant effect on the activation of this promoter. The CCAAT element may make a minor contribution to the basal promoter activity. The TATA-like elements, and presumably their associated proteins, may exert constitutive inhibitory influences on promoter I function. This could occur through an interaction with SF-1 or through the preinitiation complex, whose formation is presumably independent of the TATA boxes in this promoter. These regulatory gene structures are amenable to multifunctional control during the development of

testicular Leydig cells from fetal to adult life and at the different stages of the ovarian cycle. The essential role for SF-1 in transcriptional activation of promoter I of the prolactin receptor gene explains why PI is expressed in a tissue-specific manner in the gonads, but not in the liver and the mammary gland.

Studies on the Regulation of Androgen Synthesis. Androgen production by the Leydig cell depends on androstenedione, the immediate precursor of testosterone synthesis, and on the activity of the microsomal enzyme 17β-hydroxysteroid dehydrogenase (17HSD), that converts androstenedione to testosterone. Stimulation of the early steps in steroidogenesis leading to androgen production occurs primarily through a G_s/adenylate cyclase pathway. Previous studies have demonstrated inhibition of testosterone production in Leydig cells treated with high concentration of forskolin. Such cells exhibit increased basal and potentiated hCG-stimulated adenylate cyclase activity and cAMP production, indicating that the inhibitory action of forskolin is non-cAMP dependent and occurs beyond pregnenolone synthesis. The inhibition of steroidogenesis by forskolin appears to be related to its inhibitory action on glucose transport, since it can be mimicked by 1,9-dideoxyforskolin (1,9 DDF), a naturally occurring analog with specificity for the glucose transporter. Glucose uptake was found to be significantly enhanced in hCG-stimulated cells, and was reduced to basal levels by 1,9-DDF. Further studies demonstrated that the site of inhibition of testosterone production by forskolin and by 1,9-DDF is the 17βHSD reaction, and that this effect can be mimicked by incubating Leydig cells without glucose. Glucose-deficient media produce the same metabolic block in the absence of forskolin, with a significant reduction in 17HSD activity and testosterone production. Glucose deficiency, however, does not affect the pathway from cholesterol to androstenedione (cholesterol side chain cleavage, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase/17,20 lyase. Glucose-induced 17βHSD activation is mimicked by the addition ATP or GTP in glucose-deficient media, but not by nonhydrolyzable triphosphate analogs (AppNHp, ATPgamma-S, GppNHp), or by other nucleotides (CTP, UTP, and TTP), or NADPH.

The information obtained by using cells with intact steroidogenic pathways was corroborated by studies in which the 17β-HSD reaction was isolated from the metabolic pathway to permit controlled addition of the substrate, androstenedione. This was accomplished by using aminogluthetimide, an enzyme inhibitor that blocks cholesterol metabolism, depletes the steroid substrate from the cells, and prevents aromatization of androstenedione. These studies further emphasized the requirement for ATP for optimal androgen biosynthesis. The effects of glucose and ATP on the kinetic parameters show that the inhibition of 17βHSD in the absence of glucose results in in an increase in the apparent Km (300%) and a reduction in the apparent specificity constant (kcat/Km), whereas no significant difference in kcat was observed. However, with the addition of ATP or GTP, the apparent Km and kcat/Km of the enzyme reverts to control levels in the presence of glucose. Similarly, 1,9-DDF induces a 200% increase in the apparent Km in cells incubated in the presence of glucose, supporting the involvement of a defective glucose transporter(s) in the observed increase in apparent Km of the 17βHSD. These kinetic parameters suggest the presence of high and low affinity 17βHSD species that can be interconverted by adding or reducing glucose or ATP.

The participation of a high energy phosphorylated intermediate of 17βHSD was explored with kinase inhibitors that would block the putative phosphorylation site. KT-5720 (50%), a specific inhibitor of protein kinase A, and the calmodulin antagonist W-7 (75%) inhibit 17βHSD activity, effects that are additive, causing reductions in testosterone production to near-basal levels. This complete inhibition of 17βHSD activity suggests that multiple kinases may play a role in the activation of 17βHSD. In studies performed in the absence of Ca^{2+}, a condition under which there is presumably no calmodulin kinase activity, the addition of KT-5720 completely inhibits 17βHSD activity. The contribution of KT-5720 to inhibition of the enzyme's activity increased from 50% in the presence of calcium to 100% in the absence of calcium, which suggests compensation and/or the activation of a common 17βHSD site through different pathways. Such compensation between kinases is also suggested by the observation that basal and ATP-stimulated testosterone levels are similar in the presence and absence of calcium. It is conceivable that the activated state could reflect differences in the state of phosphorylation. Our studies suggest that the 17βHSD activation is highly compartmentalized and separate from the rest of the steroidogenic pathway, because glucose deficiency only impacts the 17βHSD reaction in steroidogenesis. The ATP generated from non-glycolytic alternative pathways in cells incubated in the absence of glucose appears to be adequate for maintaining all metabolic steps before androstenedione formation and for the reduced, but detectable levels of 17βHSD activity observed in the absence of glucose. In conclusion, the conversion of androstenedione to testosterone requires the contribution of the glycolytic pathway to meet ATP requirements for 17βHSD activity.

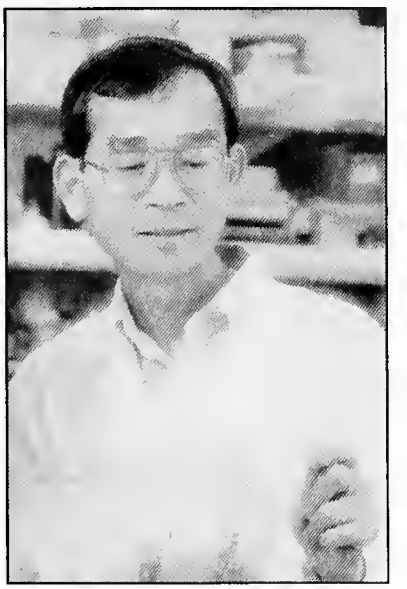
ROLE OF PROTEIN PHOSPHORYLATION AND OXIDATION IN CELL REGULATION

The Section on Metabolic Regulation, directed by ***Kuo-Ping Huang***, studies the role of protein phosphorylation and oxidation in cellular regulation. Covalent modifications of proteins by phosphorylation and oxidation play important roles in mediating many cellular events resulting from ligand-receptor interactions that generate a variety of second messengers, including cAMP, cGMP, 1,2-diacylglycerol, arachidonic acid, inositol 1,4,5-trisphosphate, Ca^{2+}, and nitric oxide. An increase in $[Ca^{2+}]_i$, either through ion channels or triggered by inositol 1,4,5-trisphosphate released from membrane phospholipids, stimulates the protein kinase C enzyme family when 1,2-diacylglycerol and free fatty acids levels are also increased. Phosphorylation of target substrates by protein kinase C has been linked to the regulation of cell growth and differentiation, secretion, cell surface receptor function, cellular metabolism, and learning and memory. In the CNS, the excitatory amino acid-mediated opening of receptor ion channels and the resulting increase in $[Ca^{2+}]_i$ also stimulates nitric oxide synthase. One of the targets of nitric oxide is the sulfhydryl group of proteins. Modification through nitrosylation, as well as disulfide bond formation, causes conformational changes in proteins and influences their biological activities.

Oxidation of Rat Brain Neurogranin in Intact Cells by Nitric Oxide and Other Oxidants. Neurogranin (Ng) is a 78-amino acid postsynaptic protein kinase C (PKC) substrate present in the neurons of the adult brain. This protein is a high affinity calmodulin (CaM)-binding protein at low levels of Ca^{2+}. Phosphorylation of Ng by PKC at a single site located within a conserved 19-amino acid homologous region of Ng/neuromodulin, which also contains the predicted CaM-binding domain, results in a weakening of its binding affinity for CaM. It has been hypothesized that the PKC-catalyzed phosphorylation of Ng and neuromodulin frees the CaM for other CaM-dependent enzymes. We have shown that rat brain Ng, which contains four cysteine residues, is readily oxidized by nitric oxide donors and by other oxidants to generate a large conformational change that is detectable on non-reducing SDS-polyacrylamide gel electrophoresis. The reduced Ng has an apparent M_r of 17 kDa, and the oxidized form has one of 10 kDa. Reversible conversion between these two Ng species was observed during treatment with oxidants, followed by reduction with sulfhydryl reagent. To investigate the physiological relevance of Ng oxidation in intact cells, we tested the effects of NO donors, oxidants [H_2O_2 and oxidized glutathione (GSSG)], and $HgCl_2$ on Ng-cDNA-transfected COS cells and on rat brain slices. Western blot analysis showed that Ng expressed in the transfected cells is readily oxidized by H_2O_2 and $HgCl_2$, but poorly by NO donors such as sodium nitroprusside (SNP) and 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEANO). Endogenous Ng in rat brain slices is more sensitive to oxidation by NO donors, and by H_2O_2, GSSG, and $HgCl_2$, than that expressed in COS cells. The SNP-induced oxidation of Ng in brain slices can be reversed by dithiothreitol, ascorbic acid, or reduced glutathione, and that induced by $HgCl_2$ by its chelator D-penicillamine. Reversible oxidation and reduction of Ng were also observed in rat brain extracts, in which oxidation is enhanced by Ca^{2+} and the oxidized Ng can be reduced by NADPH or reduced glutathione. These results suggest that the brain contains enzymes that catalyze Ng oxidation and reduction. Thus, Ng is a target not only of PKC but also of nitric oxide and other oxidants. Both the phosphorylation and oxidation of Ng could serve as signaling mechanisms for controlling the level of CaM, which is essential for many Ca^{2+}-regulated cellular processes.

Modification of Rat Brain Neurogranin by Glutathionation. Rat brain Ng contains four cysteine residues, Cys-3, Cys-4, Cys-9, Cys-51, which are all susceptible to oxidation by nitric oxide and other oxidants. Modification of Ng by nitric oxide is apparently mediated by the formation of a nitroso intermediate followed by disulfide bond formation. We found that incubation of Ng with a nitroso compound, S-nitrosoglutathione (SNOG), results in both oxidation to form intramolecular disulfide bonds and S-glutathionation. The ratio of these two modified Ng forms depends on the concentration of SNOG; the reaction favors disulfide bond formation at low concentrations, and glutathionation at high concentrations. Since SNOG undergoes rapid decomposition to generate several products including oxidized glutathione, it does not glutathionate Ng effectively. Reverse phase HPLC chromatography of decomposed SNOG resolved several components with different potencies in the glutathionation of Ng. Up to 4 mol/mol of glutathione were incorporated into Ng as determined by electrospray mass spectrometry. Glutathionated Ng, like NO-oxidized Ng, is a poorer substrate of PKC compared with Ng. However, unlike NO-oxidized Ng, it retains the ability to bind to CaM in the absence of Ca^{2+}. This novel modification of Ng by glutathionation may serve as another regulatory mechanism of Ng function. S-nitroso compounds are produced *in vivo* as a result of the interaction of NO with sulfhydryl compounds, most notably with reduced glutathione to form SNOG.

***In Vivo* States of Phosphorylation and Oxidation of Rat Brain Neuromodulin.** Neuromodulin/GAP-43 (Nm) is a PKC substrate located in the axonal growth cones of neurons. Nm and Ng are unique CaM-binding proteins that exhibit high binding affinities for CaM in the absence of Ca^{2+}. Both proteins contain a 19-amino acid homologous region that harbors the PKC phosphorylation site and CaM-binding domain. These two proteins are believed to regulate the level of free CaM in response to $[Ca^{2+}]_i$ and activation of PKC. Nm has been implicated in presynaptic events that are related to synaptic development and plasticity, modulation of neurotransmitter release, and long-term potentiation. The *in vivo* states of phosphorylation and oxidation at the two adjacent cysteine residues of rat brain Nm were investigated using electrospray mass spectrometry (ES-MS) of the purified protein. The brain homogenate was prepared in buffer containing protease inhibitors, protein phosphatase inhibitors, and a sulfhydryl group modifier to preserve the endogenous states of phosphorylation and oxidation. Several Nm species with different degrees of phosphorylation were separated by ion exchange chromatography, and nonphosphorylated, mono-, di-, tri-, and tetra-phosphorylated Nm were identified by ES-MS. Each of these Nm species contained two mol/mol of sulfhydryl modifier, such as iodoacetamide or 4-vinylpyridine, indicating that Cys-3 and Cys-4 are in the reduced form and have not been previously modified by palmitoylation. Mono- and di-phospho-Nm accounts for the majority of this protein *in vivo*, and only minute amounts of tetra-phospho-Nm were detected. The phosphorylated forms account for over 80% of the Nm in the brain. Hypoxia or ischemia causes dephosphorylation of Nm *in vivo* without affecting the oxidative state of its Cys residues. Analysis of the amino acid sequences and the masses of the peptides resulting from Lys-C endoprotease digestion identified several potential phosphorylation sites of Nm, at Ser-41, Thr-95, Ser-96 , Ser-142, and Thr-172. Among these, Thr-95 or Ser-96 and Thr-172 were found to be phosphorylated at the highest level, whereas the other sites are phosphorylated at a relatively low level. Comparing the extent of phosphorylation of these sites among mono-, di-, and tri-phospho Nm revealed that Thr-172 undergoes a higher degree of phosphorylation/dephosphorylation turnover than does Thr-95 or Ser-96. We predict that hypoxia-induced dephosphorylation of Nm is largely due to the dephosphorylation of Thr-172. Most interestingly, all these sites, with the exception of Ser-41, are not consensus phosphorylation sites of PKC. These findings indicate that several other protein kinases are also involved in the phosphorylation of Nm *in vivo*.


Kuo-Ping Huang

Promoter Activities of the 5′-Flanking Sequences of HASPP28. We recently cloned the cDNA of HASPP28, a ubiquitous phosphoprotein. Although the HASPP28 protein can be phosphorylated *in vitro* by several protein kinases, including PKA, PKC, CK1, and CK2, its phosphorylation in intact cells appears to be largely performed by a CK2-like kinase. Furthermore, both the concentration and phosphorylation of HASPP28 appear to be regulated during cell cycle progression. To elucidate the regulation of HASPP28 expression and its phosphorylation, we cloned its genomic DNA by PCR. Several overlapping DNA fragments were obtained, which collectively covered the gene spanning from + 509 bp (translation start site) upstream to -1231 bp. Most notably, there appears to be no interrupting intron in the entire coding region. The 5′-flanking sequence, -1231 to -4, was examined for the regulation of its promoter activity. A successive series of deletion constructs of the 5′ flanking sequence were fused with a luciferase reporter basic plasmid. These plasmids had been previously transfected into COS-7 cells (SV-40 transformed monkey kidney cells) and showed promoter activities. Due to human origin of the gene, and the complication of the built-in SV-40 promoter in the pGL2 plasmid, the promoter activities of these constructs were also tested in a human cell line, 293. Several observations lead to the conclusion that, although the cloned 5′-flanking sequence has a function in transcriptional regulation, it displays different characteristics in cells of different origin: as in the COS cells, plasmids pGL2/5 and pGL2/6 exhibit the highest luciferase activities; unlike in COS cells, in which they display basal activities, plasmids pGL2/7 and pGL2/8 are also active (about 80% maximum); plasmid pGL2/4 has lower activity than pGL2/3, and is less active in 293 cells than in COS cells, in which it exhibits at least 70% of maximum activity; in both cells types, plasmid pGL2/1 displays basal activity and all constructs are stimulated up to four-fold by PMA and dBu-cAMP; however, EGF is ineffective in 293 cells, unlike its action in COS cells.

Characterization of the Regulatory Elements of the HASPP28 Promoter. Several defined *cis*-acting elements, both common and unique, were identified in the cloned 5′-flanking sequence of HASPP28 gene. Most notably,

within 200 bp proximal to the translation start site (or 150 bp upstream from the putative transcription start site of -55 bp), there are separate TATA and TFIID sites, AP1/TRE sites, AP2 sites, and NFκB sites. Although all these sites are present in plasmid pGL2/1, it shows only basal activity in promoter analyses, suggesting that these sites are responsive to external stimuli. To understand how these elements function, we tested the binding of corresponding *trans*-activating protein factors using the electrophoretic gel-mobility shift assay (EMSA). End-labeled DNA fragments, -189 to -4 and -251 to -27, both containing the predicted elements of TFIID, NFκB, AP1, AP2, and a TATA box, are gel-shifted when incubated with purified p50 NFκB and TBP (37 kDa TATA box binding protein). The consensus oligonucleotide of NfκB competes for binding to p50 NFκB, but not for binding to TFIID, AP1, or AP2. The gel shift of the latter can be prevented by the unlabeled TFIID oligonucleotide, but not by oligonucleotides of NFκB, AP1, or AP2. However, purified c-jun/AP1, AP2, and SP1 proteins do not bind to any of these DNAs. A labeled double-stranded 22-mer oligonucleotide encompassing the predicted NFκB site also displays a sequence-specific shift when incubated with p50. The exact binding sites of these protein factors on the DNA were identified by footprinting analyses. The TFIID protein clearly protects the -171 to -150 sequence from DNase I digestion in all DNA fragments, which include the TATA box (tataat, -158 to -153) and the contiguous upstream AP1 site (tgactca -169 to -163). Under the same binding conditions, NFκB fails to protect any sequence from digestion. When the binding conditions of EMSA are used, p50 NFκB is able to protect three areas, namely, -181 to -168, -134 to -123, and -90 to -77, of which only the third sequence partially covers the predicted NFκB site of gtgaaacccc (-73 to -82). Interestingly, when the forward strand of DNA (-250 to -4) is labeled, p50 can be shown to protect a sequence of -88 to -66, which covers the entire NFκB site of -73 to -82. Both the EMSA and the footprinting analysis point to a functional TATA box at (-158 to -153) and imply a transcription start site roughly 30 bp down-stream of the TATA box.

ADRENAL STEROIDOGENESIS

The Section on Steroid Regulation, led by ***Charles Strott***, investigates the processes and mechanisms involved in the conjugation of steroids to highly charged chemical groups such as sulfonate. The sulfonation of biomolecules occurs widely across species, and causes dramatic changes in the physicochemical properties of the sulfonated compounds. Thus, the sulfoconjugation of steroids results in the conversion of these essentially hydrophobic compounds into more water soluble ones. While the biological importance of steroid sulfonation is poorly understood, it has several possible functions. For example, by increasing steroid polarity and thereby altering water solubility and protein binding, sulfonation can influence steroid transportability. It may also function as an initiating or termination event to produce active or inactive molecular species, and to regulate physiological responsiveness to steroids. Sulfonation, the transfer of a sulfonate group (SO_3) from a universal donor to an appropriate acceptor site, is catalyzed by sulfotransferases. One of the most active tissues in steroid sulfonation is the adrenal cortex, where members of the steroid sulfotransferase family are differentially expressed by functionally distinct cells. Current efforts to shed light on the biological consequences of steroid sulfonation include the isolation, cloning, and expression of adrenocortical steroid sulfotransferases, as well as investigation of the enzyme systems responsible for the synthesis of the universal sulfonate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS).

Isolation and Characterization of Guinea Pig PAPS Synthetase. The activation of inorganic sulfate to form PAPS is the result of the concerted action of ATP sulfurylase (reaction #1) and APS kinase (reaction #3):

(1) $ATP + SO_4^{2-} \rightarrow APS + PP_i$

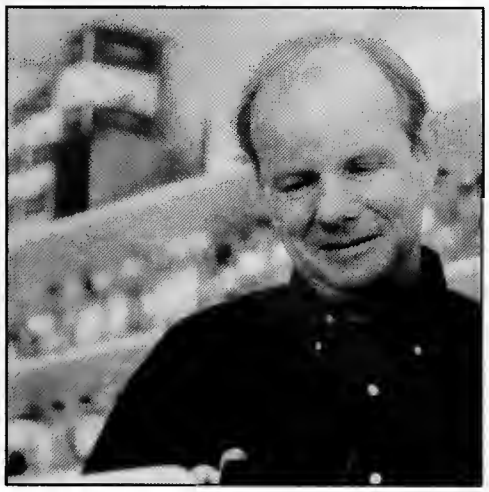
(2) $PP_i + APS \rightarrow ATP + SO_4^{2-}$

(3) $APS + ATP \rightarrow PAPS + ADP$

Reaction #1 is thermodynamically unfavorable in the forward direction; therefore, to assay for this enzyme, the more favorable reverse reaction is used (reaction #2). Thus, [^{35}S]APS and radio-inert pyrophosphate (PP_i) are used to measure the reaction by quantifying the formation of [$^{35}SO_4$]. APS kinase activity is measured by the formation of radioactive PAPS using [^{35}S]APS and ATP (reaction #3). In isolating ATP sulfurylase and APS kinase from guinea pig adrenal cytosol it was found that the two activities co-purify throughout the purification scheme, which involves S-200 gel filtration, ion-exchange chromatography and PAP-affinity chromatography. The fraction containing the coincident peak activities eluted from the affinity gel reveals a single protein band of 55 kDa when analyzed by SDS gel electrophoresis and silver staining. Isoelectric focusing of the same preparation reveals two bands with pIs of 6.8 and 6.9, suggesting charge isoforms. The bifunctionality of the

purified 55 kDa protein (PAPS synthase) is in contrast to the situation in bacteria, fungi, yeast, and plants, where ATP sulfurylase and APS kinase are associated with distinct polypeptide chains.

Cloning and Expression of Human and Guinea Pig PAPS Synthetase. Human PAPS synthase cDNA was obtained using an expressed sequence tag (EST) database developed from a directionally cloned human infant brain cDNA library, in which EST07074 was found to contain 423 bp from the 5'-end of APS kinase and to be 60% identical to yeast APS kinase. Oligonucleotide primer constructs corresponding to the EST07074 sequence were used to sequence human PAPS synthase by contiguous DNA walking until the poly(A) tail was reached. The missing 5'-upstream sequence, including the initiator ATG codon, was obtained with the RACE procedure using human fetal brain poly(A)$^+$ RNA. The guinea pig PAPS synthase cDNA was obtained using adrenal poly(A)$^+$ RNA and oligonucleotide primers based on the human PAPS synthase cDNA sequence, emploing both 5' and 3' RACE procedures. The human PAPS synthase cDNA contains 2282 bp and encodes a protein of 623 amino acids; the guinea pig PAPS synthase cDNA contains 2319 bp and encodes a protein of 624 amino acids. When human and guinea pig PAPS synthase cDNAs were expressed in COS-1 cells, Northern analysis revealed only single PAPS synthase transcripts for both human and guinea pig that are about 3.1 kb in size. This indicates that about 800 nt are missing from the 5'UTRs of the cDNAs, since the cDNAs are about 2.3 kb in size.



Charles Strott

Sequence Analyses. APS kinase gene products have been cloned from bacteria, yeast, fungi, and plants, and their deduced amino acid sequences exhibit about 30% identity and 49% homology. There are three conserved nucleotide binding motifs: a P-loop nucleotide binding motif, a GTP interactive motif, and a PAPS binding motif. The P-loop motif is variously represented as GxxxxGK (lower case x represents any amino acid), GxxxxGK(TS), GxxxxGK(S,T), GxxxxGKT/S and GxxGxGK. The GTP interactive motif consists of DxxG, and the PAPS binding motif consists of KA/GxxGxxxN/Ex(0-1)FT. The importance of these nucleotide binding motifs in APS kinase relates to their potential as functional binding sites for APS and ATP substrates. Additionally, a FISP motif, present between the P-loop and DxxG motifs, is also conserved. The importance of the latter motif relates to the suggestion that the serine in the FISP motif serves as a phosphorylated intermediate in the phosphorylation of APS to form PAPS.

Cloning of ATP sulfurylase gene products has been reported for the same organisms, but in contrast to the situation with APS kinase, the amino acid sequences of the eleven ATP sulfurylase polypeptides exhibit very little homology (less than 1%). Exclusive of bacteria, the amino acid sequence alignment shows 16% identity and 35% homology. Analysis of the alignment fails to divulge any established sequence motifs or particular regions of high homology. The alignment problem with bacteria results from the fact that ATP sulfurylase consists of two subunits in these species. PAPS synthase has now been cloned for the marine worm, *Urechis Caupo*, and for the mouse, guinea pig, and human. An amino acid alignment of the four PAPS synthase sequences reveals 69% identity and 85% homology, indicating the high degree of conservation for this crucial protein. While human PAPS synthase is 68% identical to worm PAPS synthase, it is 95% and 98% identical to guinea pig and mouse PAPS synthase, respectively. Interestingly, the three nucleotide-binding motifs as well as the FISP motif (noted above in the discussion of APS kinase) are completely conserved within the NH_2-terminal region of this bifunctional protein. In fact, it has been postulated that in mouse PAPS synthase, which contains 624 amino acids, the first 199 amino acids correspond to APS kinase activity, followed by a 37 amino acid "linker" region and then a 388 amino acid region that is equivalent to ATP sulfurylase activity. This hypothesis has been examined experimentally using human PAPS synthase, which consists of 623 amino acids. While APS kinase activity is located within the NH_2-terminal region, and ATP sulfurylase activity in the COOH-terminal section, the length of the respective active domains is extended beyond that proposed for mouse PAPS synthase. Thus, the proposed existence of a "linker" region between the two enzyme activities seems to be in question.

ATP Sulfurylase and APS Kinase "Active" Domains in PAPS Synthase. Studies were carried out using primers based on the cDNA sequence of human PAPS synthase that were designed to generate PCR products

corresponding to specific amino- and carboxy-terminal fragments. Three amino-terminal fragments (residues 1-174, 1-209 and 1-268) were constructed based on the APS kinase sequence homology information. The 1-174 amino acid construct lacks the PAPS binding motif, while the 1-209 and 1-268 amino acid constructs contain all the nucleotide binding motifs described above for APS kinase of other species. Two COOH-terminal fragments (residues 220-623 and 264-623) were constructed: with the exception of *A. thaliana*, these fragments contain minimal homology to ATP sulfurylase of fungi, yeast, and bacteria. All constructs were co-transfected into COS-1 cells along with β-galactosidase, and cellular extracts were assayed for APS kinase, ATP sulfurylase and β-galactosidase activities, as well as for the presence of either NH_2-terminal-specific or COOH-terminal-specific immunoreactive peptides.

APS Kinase Activity. Cells transfected with the 1-268 amino acid NH_2-terminal fragment of hPAPS synthase demonstrated a seven-fold increase in APS kinase activity over control cells. Furthermore, cytosolic extracts of cells transfected with this construct demonstrate an NH_2-terminal-specific immunoreactive protein band of the appropriate size (about 30 kDa). Cells transfected with the full-length hPAPS synthase cDNA also exhibit a significant increase in APS kinase activity, although the activity is only one-third of that obtained with the 268 amino acid NH_2-terminal domain construct. In contrast, cells transfected with the other two NH_2-terminal constructs (residues 1-174 and 1-209) and the two COOH-terminal constructs (residues 220-623 and 264-623) do not exhibit higher APS kinase activity than that seen for the control cells, and fail to display an NH_2-terminal-specific immunoreactive protein band.

ATP Sulfurylase Activity. Cells transfected with the full-length hPAPS synthase cDNA demonstrate a significant increase in ATP sulfurylase activity over control cells. Cells transfected with the 405 amino acid COOH-terminal segment (residues 220-623) of hPAPS synthase exhibit a significant increase in ATP sulfurylase activity that is higher than that found for the full-length preparation. These cells also produce a COOH-terminal-specific immunoreactive protein band of the appropriate size (about 47 kDa). On the other hand, cells transfected with the three NH_2-terminal constructs and the 360 amino acid COOH-terminal construct (residues 264-623) fail to exhibit an increase in ATP sulfurylase activity and do not produce a COOH-terminal-specific immunoreactive protein band.

Kinetic Analyses. The 1-268 amino acid NH_2-terminal domain of hPAPS synthase displays Michaelis-Menten kinetics for both APS and ATP substrates. Double-reciprocal transformations revealed K_m values of 0.6 μM for APS and 0.2 mM for ATP. Full-length hPAPS synthase likewise demonstrates Michaelis-Menten kinetics when tested with the APS substrate, and has a K_m of 0.4 μM. When full-length hPAPS synthase was tested against the ATP cosubstrate, a sigmoidal dose-response curve was obtained, indicating cooperative substrate binding, with an empirically-derived K_m of 1.5 mM. The latter finding suggests that the COOH-terminal domain of hPAPS synthase has a regulatory action on the APS kinase activity that is located in the NH_2-terminal domain of the protein. The APS K_m values of 0.6 μM and 0.4 μM for the NH_2-terminal domain and full-length constructs of hPAPS synthase, respectively, are similar to the APS K_m value for APS kinase of purified guinea pig PAPS synthase. In terms of catalytic efficiency, APS V_{max}/K_m for the NH_2-terminal domain is two-fold higher than for full-length hPAPS synthase, reflecting an increase in V_{max}. However, ATP V_{max}/K_m for the NH_2-terminal domain is 27-fold higher than for full-length hPAPS synthase, reflecting the increased catalytic efficiency of the NH_2-terminal domain.

Gene Fusion in Higher Eukaryotes. We next addressed the question of whether there is an adaptive advantage in the fusion of ATP sulfurylase and APS kinase into a single polypeptide chain in higher eukaryotes. While a number of advantages can be considered for the existence of multifunctional proteins in general, and for ATP sulfurylase and APS kinase in particular, the answer to the foregoing question remains obscure. The fusion protein could expedite channeling (direct transfer) of APS from ATP sulfurylase to APS kinase, an idea that takes into consideration the substantial energy barrier that exists in APS formation, as well as the instability of APS in cells. Channeling, however, can also be achieved by ATP sulfurylase and APS kinase as distinct polypeptides associating noncovalently in a multifunctional complex, as has been suggested for *E. coli*. On the other hand, filamentous fungi appear not to demonstrate channeling of APS between the two enzyme systems. Nevertheless, the development of a fused protein system could represent an evolutionary step creating a more efficient pathway that is able to overcome inherent obstacles to the synthesis of activated sulfate and production of the crucial universal sulfonate donor molecule, PAPS. In our analysis, the catalytic efficiency of the active NH_2-terminal domain of hPAPS synthase is 27 times higher than that of the complete protein, which is contrary to what would be expected if the advantage of protein fusion is to improve catalytic efficiency. Thus, at least based on the APS kinase kinetic data, it appears that the fusion of ATP sulfurylase and APS kinase does not

produce a catalytic advantage. Purified preparations of the full-length and the active COOH-terminal domain of hPAPS synthase will be required to perform kinetic analyses and further examine this question. Fusion of the ATP sulfurylase and APS kinase genes into a single gene might produce an adaptive advantage by facilitating their coordinate transcription. In *E. coli*, the coupled expression of these enzymes is accomplished by the contiguous placement of the subunits (ATP sulfurylase and APS kinase) genes within the same operon. Therefore, at first glance the fusion of ATP sulfurylase and APS kinase would not appear to have necessarily created a regulatory advantage. However, in multicellular organisms the creation of unique *cis*-elements and *trans* factors allows for differential tissue-specific expression of PAPS synthase during growth and development, as well as in response to acute and/or chronic metabolic needs.

ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

Chief
Kevin J. Catt, M.D., Ph.D.

Principal Investigators
Maria L. Dufau, M.D., Ph.D
Kuo-Ping Huang, Ph.D.
Charles A. Strott, M.D.

Tenure-Track Investigators
Stanko Stojilkovic, Ph.D.
Tamas Balla, M.D., Ph.D.

Staff Scientists
Freesia Huang, Ph.D.
Young Ja Lee, Ph.D.
Melanija Tomic, Ph.D.
Chon-Hwa Tsai-Morris, Ph.D.

Senior Fellows
Krishan Arora, Ph.D.
Azra Khanum, Ph.D.
Lazar Krsmanovic, Ph.D.
Mark Leondires, M.D.
Roger Smith, Ph.D.
Peter Varnai, M.D., Ph.D.
Ran Zhang, Ph.D.

Post-Doctoral Fellows
Harukuni Akita, M.D., Ph.D.
Leonidas Baltas, Ph.D.
Hye-Ok Chung, Ph.D.
Antonio Martinez-Fuentes, Ph.D.
Yi Geng, M.D.
Wen-Ling, Hsieh, Ph.D.
Zhang-Zhi Hu, M.D.
Taka-aki Koshimizu, M.D., Ph.D
Agnieszka Lachowicz, M.D., Ph.D
Junfa Li, M.D., Ph.D.
Nadia Mores, M.D., Ph.D.
Jesus Alberto Olivares-Reyes, Ph.D.
Jhang Ho Pak, Ph.D.
Byoung Park, Ph.D.
Kang Rao, M.D.
Suguru Saito, M.D.
Shingo Takada, M.D., Ph.D.
Pei-Zhong Tang, M.D., Ph.D.
Fredrick Van Goor, Ph.D.
Kallidaikurichi Venkatachalam, Ph.D.
Sai Xiao, M.D., Ph.D.
Qing Yang, P.H.
Meng Zhang, Ph.D.
Ying Zhang, Ph.D.
Li-Xin Zheng, M.D.
Li Zhuang, M.D.

Facility Manager
Hao-Chia Chen, Ph.D.

Guest Researchers
Massimiliano Caprio, M.D.
Gregory Downes, M.D., Ph.D.
Gowraganahalli Jagadeesh, Ph.D.
Anli Jiang, M.D.
Jianping Meng, M.D.
Carlos Navarro, M.D., Ph.D.
S. Abdul Saeed, Ph.D.
Annamaria Zolyomi, M.D.
Xiaojun Zou, M.D.

Pre-Doctoral Fellows
Suman Jayadev
Ann Chen Katzur
Cecylia Kelley

Senior Technical Specialists
Albert Baukal
John Morell

ANNUAL BIBLIOGRAPHY

Arora KK, Cheng Z, Catt KJ. Mutations of the conserved DRS motif in the intracellular loop of the gonadotropin-releasing hormone receptor affect expression, activation, and internalization. Mol Endocrinol 1997;11:1203-12.

Bagnato A, Tecce R, Di Castro V, Catt KJ. Activation of mitogenic signaling by endothelin 1 in ovarian carcinoma cells. Cancer Res 1997;57:1306-11.

Balla T. Mass assay of inositol and its use to assay inositol polyphosphates. In: Shears S, ed. Signalling by inositides. A practical approach. The Practical Approach Series. Oxford, New York, Tokyo: Oxford University Press, 1997;53-68.

Balla T, Downing GJ, Jaffe H,Kim S, Zólyomi A, Catt KJ. Isolation and molecular cloning of wortmannin-sensitive bovine type II phosphatidylinositol 4-kinases. J Biol Chem 1997;272:29:18358-66.

Cool DR, Normant E, She FS, Chen HC, Pannell L, Zhang Y and Loh YP. Carboxypeptidase E is a sorting receptor for regulated secretory pathway proteins and genetic obliteration leads to endocrinological disorders in the *Cpe*^{fat} mouse. Cell 1997;88:73-83.

Delphin C, Huang K-P, Scotto C, Chapel A, Vincon M, Chambaz E, Garin J, Baudier J. The *in vitro* phosphorylation of p53 by calcium-dependent protein kinase C, characterization of a protein-kinase-C-binding site on p53. Eur J Biochem 1997;245:684-92.

Dufau ML, Miyagawa Y, Takada S, Khanum A, Miyagawa H, Buczko E. Regulation of androgen synthesis: the late steroidogenic pathway. Steroids 1997;62:128-32.

Hu Z, Zhuang L, Guan X, Meng J, Dufau ML. Steroidogenic Factor-1 is an essential transcriptional activator for gonad-specific expression of promoter I of the rat prolactin receptor gene. J Biol Chem 1997;272:14263-71.

Hunyady L, Zhang M, Jagadeesh G, Bor M, Balla T, Catt KJ. Dependence of agonist activation on a conserved apolar residue in the third intracellular loop of the AT_1 angiotensin receptor. Proc Natl Acad Sci USA 1996;93:10040-5.

Johnson BE, Damodaran A, Rushin J, Gross A, Le PT, Chen HC, Harris RB. Ectopic production and processing of atrial natriuretic peptide in a small cell lung carcinoma cell line and tumor from a patient with hyponatremia. Cancer 1997;79:35-44.

Khanum A, Buczko E, Dufau ML. Essential role of adenosine triphosphate in activation of 17β-hydroxysteroid dehydrogenase in the rat Leydig cell. Endocrinology 1997;138:1612-20.

Kukuljan M, Vergara L, Stojilkovic SS. Modulation of the kinetics of $InsP_3$-induced $[Ca^{2+}]_i$ oscillations by calcium entry in pituitary gonadotrophs. Biophys J 1997;72:698-707.

Lachowicz A, Van Goor F, Katzur AC, Bonhomme G, Stojilkovic SS. Uncoupling of calcium mobilization and entry pathways in endothelin-stimulated pituitary lactotrophs. J Biol Chem 1997;272:28308-14.

Li YX, Stojilkovic SS, Keizer J, Rinzel J. Sensing and refilling calcium stores in an excitable cell. Biophys J 1997;72:1080-91.

Mahoney CW, Pak J, Huang K-P. Nitric oxide modification of neurogranin: identification of the cysteine residues involved in intramolecular disulfide bridge formation using site-directed mutagenesis. J Biol Chem 1996;271:28798-804.

Martìnez A, Miller MJ, Catt KJ, Cuttitta F. Adrenomedullin receptor expression in human lung and in pulmonary tumors. J Histochem Cytochem 1997;45:159-64.

Rao K, Paik W-K, Zheng L, Jobin RM, Tomic M, Jiang H, Nakanishi S, Stojilkovic SS. Wortmannin-sensitive and -insensitive steps in calcium-controlled exocytosis in pituitary gonadotrophs. Endocrinology 1997;138:1440-9.

Reinhart J, Xiao S, Arora KK, Catt KJ. Structural organization and characterization of the promoter region of the rat gonadotropin-releasing hormone receptor gene. Mol Cell Endocrinol 1997;130:1-12.

Rössig L, Zólyomi A, Catt KJ, Balla T. Regulation of angiotensin II-stimulated Ca^{2+} oscillations by Ca^{2+} influx mechanisms in adrenal glomerulosa cells. J Biol Chem 1996;271:22063-9.

Sheu F-S, Mahoney CW, Seki K, Huang K-P. Nitric oxide modification of rat brain neurogranin affects both the phosphorylation by protein kinase C and the affinity for calmodulin. J Biol Chem 1996;271:22407-13.

Stojilkovic SS, Zheng L, Tomic M, Cesnjaj M, Jobin RM, Catt KJ. GnRH-induced intracellular signaling in pituitary gonadotrophs. In: Weixiong L, Li RM, Chen H-C, Hahn DW, eds. Fertility regulation: present and future. Proceedings of the Beijing International Symposium on Fertility Regulation. Washington, DC: US DHHS, 1997;2:89-103.

Strott CA. Steroid sulfotransferases. Endocr Rev 1996;17:670-97.

Vergara L, Rojas E, Stojilkovic SS. A novel calcium-activated apamin-insensitive potassium current in pituitary gonadotrophs. Endocrinology 1997;138:2658-64.

Zheng L, Krsmanovic LZ, Vergara LA, Catt KJ, Stojilkovic SS. Dependence of intracellular signaling and neurosecretion on phospholipase D activation in immortalized gonadotropin-releasing hormone neurons. Proc Natl Acad Sci USA 1997;94:1573-8.

HERITABLE DISORDERS BRANCH

Joan C. Marini, M.D., Ph.D., Chief

Members of the Heritable Disorders Branch perform both clinical and basic research into the etiology, diagnosis, and therapy of human genetic diseases. Clinically, inborn errors of metabolism and connective tissue disorders in children are especially emphasized. Clinical investigators admit patients to the pediatric ward of the NIH Clinical Center under protocols to study rare diseases. These disorders are chosen both for the benefit that can be offered to affected patients, and because identifying the cause of a specific dysfunction often unveils a normal process. All the clinical members of the Branch also conduct basic research on their diseases of interest in addition to providing clinical care. The basic research groups in the Branch investigate molecular or biochemical aspects of human genetic disease, and interact closely with their clinical counterparts on a technical level.

CONNECTIVE TISSUE DISORDERS

The Section on Connective Tissue Disorders, directed by ***Joan Marini***, conducts a fully integrated program of laboratory and clinical research, aimed at characterizing the molecular mechanisms of the heritable connective tissue disorders osteogenesis imperfecta and Ehlers-Danlos Syndrome, and at applying the information to the diagnosis and treatment of affected patients.

Molecular Therapeutics for Osteogenesis Imperfecta. OI is a dominant disorder of connective tissue, in which affected individuals have fragile bones susceptible to fracture from minimal trauma. OI is caused by mutations in type I collagen, the most abundant structural protein in the body. In general, mutant chains are incorporated into the collagen helix, secreted from the cell and integrated into the extracellular matrix. Thus, a gene therapy approach aimed at adding a normal collagen gene would not be expected to be effective in such a dominant negative disorder. A major interest of this Section is to develop allele-specific, selective suppression of the mutation-carrying mRNA as an approach to therapeutic intervention. Recently, we have focused on hammerhead ribozymes. The combination of requirements of the ribozyme for both a binding site and a cleavage site enhances the specificity and efficiency of cleavage in comparison with linear oligonucleotides.

We have studied hammerhead ribozymes *in vitro* and have demonstrated the absolute allele-specificity of cleavage. Ribozymes were targeted against synthetic transcripts of naturally occurring human collagen mutations, as well as against a point mutation introduced into a murine collagen construct. Only transcripts containing the mutation-induced ribozyme cleavage site are cleaved. Normal RNA remains intact, even though it carries the complete ribozyme binding sequence. We demonstrated an approximate doubling of the percent of product obtained with each five-fold increase in ribozyme/substrate ratio in the range of 1:1 to 25:1; higher ribozyme/substrate ratios are saturating. The competitive effects of normal transcripts on ribozyme cleavage activity were investigated. Cleavage is inhibited by the presence of non-cleavable competitor substrate in a linear fashion, as would be the case if both mutant and normal transcripts were present in the cell. *In vitro*, this competition can be eliminated by the introduction of a mismatch into one ribozyme binding arm.

More recently, we have initiated experiments to determine the effectiveness and specificity of hammerhead ribozymes *in cellulo*. The cell lines to be employed for this purpose have collagen mutations that simultaneously also generate a novel ribozyme cleavage site and change a restriction site. Primary skin fibroblasts have been stably transfected with a pCI.neo mammalian expression vector harboring an active or inactive ribozyme template. RT-PCR analysis confirmed the expression of the ribozyme transcripts. In preliminary experiments, we found that the ratio of mutant to normal collagen mRNA is significantly decreased in cells harboring active ribozyme, compared with cells harboring inactive ribozyme.

Development of a Non-Lethal Murine Model for OI. Currently, no animal model for OI is available that is non-lethal, has significant physical manifestations, and expresses a mutation similar to a naturally occurring collagen mutation. In cooperation with the LMGD, this Section is engaged in a project to generate a conditional non-lethal murine model for OI. The causative collagen mutation produces a glycine substitution in $\alpha 1(I)$, a substitution that is typical for severe human OI. The Section has used gene targeting and homologous

recombination in embryonic stem cells to create the first murine knock-in model for OI. The construct bears two crucial elements: (1) a G → T change was introduced in exon 23 at nt 1546, a mutation previously characterized by this Section in a case of human type IV OI, which causes the substitution gly349cys; (2) since a severe OI phenotype could compromise the reproductive success of the affected mice, a transcription/translation "stop" cassette containing an alternative splice donor site and an AUG codon, flanked by two direct loxP elements, a sequence of 34 bp recognized by cre recombinase, was inserted into intron 22. It was expected that the F1 would have null expression of the mutant allele, as associated with mild OI. Afterwards, mating of the F1 generation with mice expressing cre under the control of the EIIa promoter is predicted to yield an F2 with expression of OI in a phenotypically severe non-lethal form.

The construct was electroporated into 129SV ES cells and three male chimera were generated. The chimera were used to generate two murine modes with a skeletal phenotype characteristic of human OI. The first knock-out, generated from a chimera x wt mating, was lethal due to the alternative splicing generated by the presence of the lox-stop-lox itself, since one of the four alternatively spliced forms was in-frame. Radiography, histology, and skeletal staining confirm a phenotype with multiple rib fractures and poor skeletal mineralization. No mutant protein was secreted and the phenotype appears to result from protein suicide effects. The chimeras have also been mated with mice expressing cre recombinase, to eliminate the cassette. About half the pups carrying the mutant allele underwent cassette excision. The mutant allele was expressed and dimers of the mutant α1(I) chains were detected. Thus, the cre/lox system functioned successfully to yield expression of the mutant allele. This opens the possibility of using cre/lox to generate animal models for other dominant disorders with severe/lethal forms.

Molecular Characterization of OI Bone. To gain insight into the underlying pathobiology of this bone disease, this Section studies the molecular and biochemical characteristics of cultured OI osteoblasts. The type I collagen synthesized by osteoblasts and fibroblasts has been electrophoretically analyzed in patients affected with type IV OI. Previous work with steady-state labeling of collagen had shown that the collagen produced by osteoblasts is more overmodified than collagen produced by the fibroblasts from the same patient. More recently, this lab examined the synthesis, secretion, and matrix incorporation of collagen from two additional type IV OI patients, one with an α1(I) gly832ser substitution and one with an α2(I) exon 16 splicing defect. For the proband with the splicing defect, mutant chains are detectable in the cells after a short labeling pulse but are poorly secreted and are barely detectable in the steady-state media sample. For the glycine substitution, mutant chains appear more slowly in the cell but are well secreted and are abundant in steady-state media samples. When the matrix deposited by long term cultures was examined, the mutant chain with abnormal splicing could be detected in bone matrix but tends to accumulate in the immaturely processed form. The mutant chains with the glycine substitution are well-deposited in all fractions of matrix. Thus, although the probands both have type IV OI, their collagen matrix composition is different, suggesting a complex relationship between matrix composition and phenotype.


Joan Marini

Mutations in OI Patients. This Section has continued to identify the collagen protein abnormalities and associated molecular defects in patients with osteogenesis imperfecta. We have previously proposed a Regional Model for the relationship between clinical severity and mutation location. The new mutations, which have been delineated along the α2(I) chain, are compatible in location with the Regional Model, in which there are three lethal and four non-lethal regions alternating along the chain. This year, the Section has delineated two novel mutations in NIH patients, α2 gly250ser and α2(I) gly703asp, which are located in non-lethal regions and are compatible with the genotype/phenotype relationships of the Regional Model.

The Section has also identified a mutation that extends the phenotype associated with structural mutations in type I collagen. Two siblings, diagnosed with juvenile osteoporosis, have physically and radiographically detectable lesions only in the spine, but no long bone fractures. Use of RNA hybrid analysis identified a G → A change in both siblings, causing a gly436arg substitution. This case also suggests that certain regions of collagen may be crucial only in specific skeletal elements.

Clinical Investigation of OI. This Section has continued its investigation of the clinical aspects of OI, such as growth deficiency, neurological abnormalities, and physical rehabilitation. The growth hormone treatment trial, in which 26 children with types III and IV OI were treated with growth hormone, is nearing its conclusion. The responder group is composed predominantly of children with type IV OI. The only bone-metabolic marker that distinguishes responders and non-responders is type I collagen peptide. The bone histomorphometrics of responders show a significant increase in the ratios of bone volume/total volume and of mineralized bone volume/total volume and in the bone formation rate. It appears that there is a significant population of OI children, especially with type IV OI, who respond to growth hormone treatment with increased growth rates and beneficial changes in bone formation.

INBORN ERRORS OF METABOLISM

The Section on Human Biochemical Genetics, under the direction of ***William Gahl***, studies the clinical, biochemical, and molecular aspects of rare biochemical disorders.

Gene for Nephropathic Cystinosis: Narrowing the Search. Members of the Section have a long tradition of offering new insights into the understanding of the autosomal recessive disorder nephropathic cystinosis. Fifteen years ago, this group demonstrated that in cystinosis the disulfide amino acid cystine fails to be transported out of lysosomes, which results in poor growth, renal failure by age 10 years, and a variety of parenchymal organ disorders due to cystine accumulation. After helping to show the clinical efficacy of cysteamine with respect to growth and prevention of renal deterioration, members of the Section are now following approximately 100 cystinosis patients with semiannual surveillance. The intent is to determine whether children treated with cysteamine can avoid the nonrenal complications of cystinosis, and for how long renal failure can be averted. In collaboration with investigators in the National Eye Institute, members of the Section also provide patients with cysteamine eye drops to dissolve the painful corneal cystine crystals characteristic of this disorder. Finally, the Section remains a resource for information and advice concerning this rare, formerly fatal, disease.

Now, the Section is also making important contributions toward the search for the cystinosis gene. We previously led a consortium, which mapped the cystinosis gene by linkage analysis to a 3.1 cM region of chromosome 17p13 flanked by the microsatellite markers D17S1828 and D17S1798. After identifying a yeast artificial chromosome (YAC) contig spanning the cystinosis region (in collaboration with David Ledbetter and Akira Tanigami of the NHGRI), we identified a new polymorphic microsatellite repeat marker, D17S2167, in the critical region. Recombination analysis demonstrated that the cystinosis gene lies between D17S2167 and D17S1828, and P1 clones containing these markers have been identified.

These flanking P1s were end-sequenced to create new sequence-tagged sites (STSs), which serve as probes for the construction of a bacterial artificial chromosome (BAC) contig across the cystinosis critical region. The contig is being created in collaboration with Eric Green and Jeff Touchman of the NHGRI. BACs containing an STS located on one of the two flanking P1s (as determined by PCR amplification) are end-sequenced. STSs are formed from these end sequences and used to reprobe a BAC library for new BACs, which are themselves end-sequenced, etc. In this fashion, the critical region of the cystinosis gene is bridged by overlapping BACs, creating a contig. The direction of the contig can be verified by determining shared STS markers and by radiation hybrid analysis. To date, over 25 nonpolymorphic STSs from approximately 10 BAC and 2 P1 clones have been mapped within the cystinosis interval.

Once the contig is complete, its components will be sample-sequenced. This procedure has already been performed for the two flanking P1s. In this technique, BAC or P1 clones are converted to M13 libraries, from which random clones are sequenced to an estimated redundancy of 1.5-2.0. The sequence is compared with those in public databases to identify expressed sequence tags (ESTs). These are the 3′ ends of cDNAs. To date, five genes have been mapped to two genomic clones in the cystinosis region. These and other candidate genes will be tested to determine if one is the cystinosis gene by using them as probes against Northern blots containing RNA from a variety of cystinosis patients. The size or amount of the cystinosis gene mRNA from an occasional patient should be abnormal, and this will be detected if the probe is the cystinosis gene itself. The cystinosis gene would be the first lysosomal membrane protein with a known function to be cloned and sequenced.

Hermansky-Pudlak Syndrome. The Section's other main pursuit is an understanding of Hermansky-Pudlak syndrome (HPS), another autosomal recessive disorder consisting of oculocutaneous albinism, a bleeding

diathesis due to absent platelet dense bodies, and lysosomal storage of ceroid lipofuscin, an amorphous lipid-protein substance, which can cause pulmonary fibrosis and granulomatous colitis in HPS. The pulmonary disease often proves fatal in the fourth or fifth decades of life. HPS is common in a northwest Puerto Rican isolate, where an HPS gene was mapped to chromosome 10q23 and its cDNA cloned and sequenced. The gene, *HPS*, has no homology to known sequences, and produces a 79.3 kDa protein of unknown function. In the original report on this gene, all Puerto Rican patients with HPS were found to be homozygous for a 16-base pair duplication in exon 15.

Since August of 1995, we have studied 49 HPS patients age 3 to 54 years on a clinical and molecular level to better characterize HPS among Puerto Ricans and non-Puerto Ricans. Of 27 Puerto Rican patients, 25 are homozygous for the 16 bp duplication; none of 22 non-Puerto Rican patients exhibit the 16 bp duplication in either allele. The 25 duplication patients (mean age 24 years) and 24 non-duplication patients (mean age 19 years) do not differ in their wide range of skin, hair, iris, and fundus pigmentation, or in their visual acuities (20/320 to 20/50). They also exhibit equivalent frequencies of major bleeding and of colitis. In addition, a variety of laboratory tests, including blood cell counts and coagulation times, liver function tests, thyroxine levels, and other blood chemistry values, were found to be normal in both groups. However, compared with the 10 non-duplication adults, the 16 adults homozygous for the duplication have lower mean forced vital capacity (82 $\pm$ 5 SE % of predicted vs 100 $\pm$ 4%), total lung capacity (85 $\pm$ 5% vs 96 $\pm$ 3%), FEV_1 (86 $\pm$ 5% vs 104 $\pm$ 4%), and DLCO (82 $\pm$ 6% vs 108 $\pm$ 4%), FEV_1 and DLCO being measures of pulmonary function (the first how fast one can expire and the second, how well carbon monoxide gets from the lung space into the blood). These differences are significant at $p < 0.05$, and are supported by findings on thin-section, high-resolution CT scans of the lung. The duplication patients also have a lower mean creatinine clearance compared with the non-duplication patients (101 $\pm$ 5 SE vs 113 $\pm$ 6 ml/min/1.73m^2) as well as a lower mean percentile for cholesterol, i.e., 48% (175 $\pm$ 7 mg/dl) vs 65% (186 $\pm$ 8 mg/dl). These findings indicate that homozygosity for the 16 bp duplication in *HPS* is associated with severe pulmonary disease and, perhaps, mild renal glomerular impairment.


Bill Gahl

We have initiated a therapeutic protocol designed to determine the safety and efficacy of corticosteroids or pirfenidone, an anti-fibrotic agent, in treating the pulmonary fibrosis of HPS. The protocol received Institutional Review Board approval in February of 1997, and now awaits approval by the Office for the Protection of Research Risks.

In molecular studies, two individuals from central Puerto Rico lacked the 16 bp duplication found in patients from northwest Puerto Rico. Both exhibit normal amounts and size of *HPS* mRNA by Northern blot analysis. Their haplotypes in the HPS region are different from the haplotype of every 16-bp duplication patient. Moreover, they display no mutations in their cDNA sequences throughout the entire *HPS* gene. Yet they display pigment dilution, impaired visual acuity, nystagmus, a bleeding diathesis, and are lacking platelet dense bodies, confirming the diagnosis of HPS. These findings mean that HPS is most likely heterogeneous with respect to gene locus, and this is consistent with the existence of several mouse strains manifesting both pigment dilution and a platelet storage pool deficiency. The data also indicate that a diagnosis of HPS cannot be based on finding the 16-bp duplication alone.

In non-Puerto Rican patients, single strand conformation polymorphism (SSCP) analysis and sequence analysis have identified different mutations in *HPS*. However, several affected individuals have no evidence of a mutation in *HPS*, confirming the disorder's presumed locus heterogeneity. In our pursuit of the basic defect in HPS, we have created an *HPS* construct tagged with *myc* and multiple histidine markers detectable by fluorescent probes, which is being used to determine the intracellular location of *HPS* expression. A yeast two-hybrid system is also being employed to identify protein(s) that bind to the *HPS* protein in the cell's cytoplasm. Because the disease HPS involves the melanosome, lysosome, and platelet, the *HPS* gene is probably involved in the genesis or movement of these organelles' membranes. Hence, discovering the function of *HPS* and other genes causing HPS should elucidate important aspects of intracellular vesicular trafficking.

DRUG DETOXIFYING ENZYMES

The Section on Genetic Disorders of Drug Metabolism, under the direction of ***Ida Owens***, studies the regulation of the UDP glucuronosyltransferase (transferase) gene family at the molecular level using the human system. Several transferase isozymes catalyze critical substrates such as bilirubin, bile acids, steroids, natural food components, food additives, and exogenous toxins; the family of glucuronidating (detoxifying) enzymes is present in the liver, as well as in nonhepatic tissues. The aim of Owens' program is to isolate and characterize the genes and cDNAs encoding these isozymes.

Extension of the UGT1 Locus. We have essentially completed the description of the UGT1 locus. Its characterization has been critical to determining defects in patients with Crigler-Najjar (CN) diseases. The original version of the gene complex, *UGT1A-UGT1*F and recently renamed *UGT1A1-UGT1A6*, encodes one bilirubin-, three bilirubin-like-, and one phenol-UDPglucuronosyltransferase. In the 5′ region of the locus, six different exons 1 (including one pseudo gene), each with an upstream promoter and each encoding the amino terminus of an isoform, are arrayed in series with four common exons encoding five identical carboxyl termini in the 3′ region. The exon in the most 5′ position, *UGT1A6*, of the original version of the locus was used to select other cosmid clones to extend the locus by more than 310 kb. We identified, sequenced, and mapped six additional exons 1 encoding UGT1A-UGT1A12P. To search for additional exons 1 and to determine whether exons from other transferase families are co-mingled with exons 1 in the A family, we developed a highly conserved sequence (33-mer) near the 3′ end of certain exons 1, or its equivalent in family B, as a universal probe. We have begun to use the long Bac and Pac clones to overlay and connect cos clones at this locus. Using the universal probe, we have identified an additional 13 exons: two resembling 1A7-1A12 and eleven exons that belong to an entirely new cluster. With the extension, the locus is at least 500 kb long with two islands to be connected.


Ida Owens

Nomenclature of the UGT1 Locus. The novelty of this locus created confusion among the Human Nomenclature Committee and others as to whether the locus represents single or multiple genes. An agreement has been reached, consistent with the designation by our group of the original locus, that the locus represents multiple genes of the UGT1 family of transferase isozymes, each having independent regulation but linked through the 3′ end. To be consistent with the nomenclature for other transferase families and in agreement with other researchers in the field, we took the lead in renaming the genes in the UGT1 complex. The genes previously designated *UGT1A* through *UGT1F* will be renamed *UGT1A1* through *UGT1A6* in this document and in future publications. In the extended portion of the locus, newly identified exons 1 representing genes will continue with the nomenclature: *UGT1A13* etc., to be renamed according to position from 3′ to 5′. Mutant alleles for the bilirubin transferase will be designated *UGT1A1**2 etc., based on the order of discovery of the alterations in the exon 1 of the *UGT1A1* gene. Alleles with mutations in a common exon will be designated *UGT1**1, *UGT1**2 etc., demonstrating an effect on the entire UGT1 locus.

In order to expedite the sequencing and the arrangement of the exons at this locus, we have applied to the NIH Intramural Sequencing Center for sequencing of the clone with the seven new exons, as well as of a shortened version of Bac and Pac clones with two new exons each.

Synthesis of cDNAs Encoding Newly Discovered Genes and Substrate Specificity of the Isoforms. In order to expedite the determination of substrate specificity of these newly uncovered and encoded isoforms, we constructed the equivalent cDNAs for expression in COS-1 cells. Since a cDNA, HLUG P4, encoded by *UGT1A9* had already been reported in the literature, we isolated the same cDNA from a hepatocyte cDNA-lambda-ZAP library to use in the construction of cDNAs representing the *UGT1A7*, *UGT1A8*, and *UGT1A10* genes. Overlaps between the exon 1 of a gene and a common fragment from the *UGT1A9* cDNA were made by using polymerase chain reaction-amplified material. The overlap was extended by the Klenow fragment to generate the unique 5′ terminus and a portion of the common end of the new cDNA. This strategy was

successful because the 3′ end of exon 1 of *UGT1A7-UGT1A10* genes contains a common 60 nucleotide sequence (the overlap) that is contiguous with the identical 3′ common end (738 nt) of all genes encoded at this locus. Hence, the 798 bp-3′ ends of the *UGT1A7/10* cDNAs are predicted to be identical. The constructions of the cDNAs were required since we were unable to select these particular cDNAs from a human hepatocyte cDNA-lambda-ZAP library. Two cDNAs representing exons 1 of *UGT1A3* and *UGT1A5* uncovered during the initial characterization of the *UGT1* locus have been constructed in a similar fashion, using the *UGT1A4* cDNA (HUG-Br2). All cloned isoforms are undergoing analysis for substrate specificity.

We have shown that the *UGT1A8* cDNA-based protein, when expressed in the COS-1 cells, glucuronidates a new class of acceptor substrates. The rank in substrate turnover is flavones > anthroquinones > 17(-ethynyl-estradiol > complex alkyl phenols > biphenyls > bilirubin > benzo[a]pyrenes > α-naphthol. Unlike other transferase isozymes, we showed that this isozyme has only a low pH optimum.

Mutations in the Simple Phenol Transferase Isoform. For the first time, we found missense mutations in exons that code for phenol-selecting domains, two in one allele of the *UGT1A6* gene of a human genome creating heterozygosity. The mutant allele, *UGT1A6*2*, contains T181A and R184S substitutions, which create BsmI and BsoFI endonuclease sites respectively. We verified heterozygosity by BsmI- and BsoFI-sensitivity of PCR-amplified genomic DNA from this individual. Upon screening some 98 individual DNA samples, we discovered 33 individuals who are also heterozygous for the two mutations on one allele. One of these individuals also carries the R814S mutation on the second allele. Manifestations of drug sensitivity or reactions by this individual could not be ascertained. We predict that 2/100 individuals are homozygous for the two linked mutations. We also measured acceptor substrate activities with the UGT1A6 and the UGT1A6 variant (A+ and S+) isoforms using chemicals of seven different categories. We found, for the first time, that both isozymes glucuronidated 1.1 to 6-fold better at pH 6.4 than at the typical pH 7.6 assay condition. The rank of chemical turnover for the UGT1A6 isoform is: simple phenols > coumarins > complex alkyl phenols > α-naphthol > acetaminophen. Among therapeutic drugs, glucuronidation of salicylic acid derivatives, dopa derivatives, and β-blockers by UGT1A6*2 is reduced 50-75 %. Analysis of the data shows that while size and bulkiness are factors that restrict glucuronidation by the isoforms, the pKa value of the hydroxy group of all substrates is 7.8 and above. The UGT1A6 protein and its rat homolog were previously shown by other researchers to glucuronidate acetaminophen.

TATA Box Mutations in the Bilirubin Transferase Gene. A new class of mutations at the *UGT1* locus involving the TATA box transcriptional element of the *UGT1A1* gene was found by this group to exist as heterozygous genotypes for both CN-I (CF) and CN-II (SM/TS) individuals; each patient's genome bears a second missense mutation in the coding region. An allele contains either a TA deletion or TA insertion at the natural [A(TA)7A] TATA box element of both the CN-I and the CN-II patients. With the pCAT reporter gene fused to the A1 upstream regulatory region (-1.7 kb) containing either a wild-type [A(TA)7A], a TA-deleted [A(TA)6A], or a TA-inserted [A(TA)8A] at the TATA box, expression in HepG2 cells showed that the transcriptional rates were 65% and 20% normal, respectively, for the TA6 and TA8 mutants. In the coding region of these compound heterozygotes, the mutations I294T (SM) and R336W(CF) reduce glucuronidation rates to 22 % and 3% of normal, respectively. From these *in vitro* results, it is predicted that the level of bilirubin transferase activity contributed by the two alleles of SM (CN-II) and CF (CN-I) is 48% and 3% of normal, respectively. We discovered a liver-specific DNA binding protein that binds only to the mutant TATA box and not to the wild-type box.

The evidence presented here on CN diseases and recently published data on patients suffering from Gilbert's disease show that a single genetic defect (TATA box mutations) can exist among both of the CN diseases and the Gilbert's syndrome. Hence, the severity of the phenotypes depends upon the additive effect of at least two mutations, whether homozygous or compound heterozygous. We believe that the inheritance, previously considered unclear, of all the bilirubin-related diseases can be accounted for by recessive combinations of relative deleterious defects at the UGT1A1 gene culminating in the phenotypes (CN-I, CN-II, and Gilbert's) observed in the population.

Structure-Function Relationship in the UGT1A1 Protein. Using the RAO-ARGOS computer program designed to detect buried helices in membrane-anchored proteins, we discovered that the membrane-bound UGT1A1 protein contains a buried helix in microregion A, a structure shown to be unique to bilirubin isoforms. Peak hydrophobicity centers around the 170/171 di-phenylalanine. Prompted by the unique hydrophobic characteristics of this region in the UGT1A1-type proteins, we made select amino acid changes in this region

and analyzed for structure-function relationship of the substitutions. The RAO-ARGOS program reflected effects of amino acid substitutions at 170/171 and of other hydrophobic residues in the region on the buried helix. Mutant proteins with Phe-170 changed to Leu, Val, Ala, Tyr, or Glu display between 0 and 10 % of normal activity at pH 6.4, but 14 to 65 % of normal activity at pH 7.6, while those with Phe-171 changed to Leu, Val, Ala, and Tyr display between 38 and 51 % of normal activity at pH 6.4 and higher values at pH 7.6; Leu-171 is completely normal. Ile-170 or Ile-171 displays no inactivity at pH 6.4 and 20 % activity at pH 7.6. Although certain substitutions at position 170 satisfy the hydrophobicity requirement, Phe-170 is not replaceable. No doubt, Phe-170 is essential for the appropriate helical structure in this region. This hydrophobic region, which is critical for bilirubin transferase activity, reflects the most hydrophobic milieu in the protein, presumably, for binding to or interacting with the extremely hydrophobic bilirubin substrate.

GLYCOGEN STORAGE DISEASE AND METHIONINE ADENOSYLTRANSFERASE DEFICIENCY

The Section on Cellular Differentiation, led by ***Janice Chou***, seeks to understand the molecular genetics of glycogen storage disease type 1 (GSD-1) and methionine adenosyltransferase (MAT) I/III deficiency. The Section has established the molecular basis of GSD-1a and developed a gene-based diagnosis of this disorder. We have also generated *G6Pase*-deficient mice that mimic the pathophysiology of human GSD-1a patients. The null *G6Pase* mice provide a valid animal model to study the pathogenesis of GSD-1a, to delineate the mechanisms of G6Pase catalysis, and to develop future therapeutic approaches. We have also established the molecular basis of MAT I/III deficiency, developed a gene-based diagnosis for this disorder, and demonstrated that a complete lack of MAT I/III activity leads to neurological abnormalities.

Glycogen Storage Disease Type 1. GSD-1 is caused by a deficiency of glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis. This metabolic disease is characterized by fasting hypoglycemia, hepatomegaly, growth retardation, enlarged kidneys, hyperlipidemia, hyperuricemia, lactic acidemia, and in adults, a high incidence of renal failure and hepatic adenomas. We have characterized the *G6Pase* cDNAs and genes, identified a large number of mutations in the gene that abolish or greatly reduced enzymatic activity, and developed a gene-based diagnosis of GSD-1a. Using SSCP analysis and DNA sequencing, we have characterized the *G6Pase* gene in nine Israeli, in two Muslim Arab, and in two Hungarian GSD-1a patients. We showed that R83C is the prevalent mutation in Ashkenazi Jews, which can be used as an initial screening for Ashkenazi Jews suspected of having GSD-1a. We also discovered three new mutations, V166G, E110Q, and D38V. The V166G and D38V mutations abolish G6Pase activity, but the E110Q mutant retains 87% of wild-type G6Pase activity.

Janice Chou

Patients afflicted with GSD-1a cannot maintain glucose homeostasis. Current treatments consist of dietary supplementation with nocturnal nasogastric infusion of glucose or orally administered uncooked cornstarch. While this approach can maintain normoglycemia, it cannot maintain normal serum levels of lactate, cholesterol, and triglyceride; kidney function eventually becomes compromised. At present, it is unclear whether other long term complications, such as gout, hepatic adenomas, and osteoporosis can be prevented by dietary intervention alone. Thus, the transfer of a functional *G6Pase* gene to the liver of these patients may be beneficial for long-term therapy. As a first step towards the development of hepatic gene therapy for GSD-1a, we have generated a G6Pase-deficient animal model and shown that the G6Pase$^{-/-}$ mice manifest essentially the same pathophysiology as human GSD-1a. We chose the adenoviral vectors to deliver the *G6Pase* gene because adenoviruses can efficiently infect dividing and nondividing cells, propagate high titer viral stocks, and express high levels of the gene product. To date, we have constructed a mouse with G6Pase-bearing recombinant adenovirus; the adenovirus carries both an E1 deletion and *ts*E2a (Av2-mG6Pase). In collaboration with J.M. Wilson at the University of Pennsylvania, we are now constructing a mouse *G6Pase*-bearing recombinant adenovirus carrying E1 and E4 deletions (Av3-G6Pase). Preliminary experiments indicate that the Av2-mG6Pase

can efficiently deliver the *G6Pase* gene into the G6Pase$^{(-/-)}$ hepatocyte lines that we recently established, and into the liver of the mouse carrying the *G6Pase* null mutation.

We have employed the GSD-1a mice to characterize the G6Pase system, which is thought to consist of a G6Pase catalytic unit and associated translocases. By examining G6P transport, G6P hydrolysis, and pyrophosphate transport in liver and kidney microsomes from G6Pase$^{+/+}$ and G6Pase$^{-/-}$ mice, we showed that the knockout of the *G6Pase* gene destroys both G6Pase activity and G6P transport, demonstrating a tight coupling between the two reactions. We also showed that G6P transport and hydrolysis are performed by separate proteins. For pyrophosphate transport, we demonstrated that the newborn mouse kidney is more efficient than the liver, and both tissues are more efficient than adult tissues. Moreover, unlike G6P transport and hydrolysis, which are tightly linked, pyrophosphate transport is independent of other elements of the G6Pase system. These studies clearly demonstrate the involvement of multiple components in G6Pase catalysis.

We examined *G6Pase* mRNA expression, G6Pase activity, G6P uptake in both liver and kidney during normal mouse development. In the liver, *G6Pase* mRNA and enzymatic activity are low in the embryonic stages, only detected at 18 days gestation, then increase markedly at parturition before leveling off to adult levels. In the kidney, *G6Pase* mRNA and enzymatic activity were detected at 19 days gestation, increasing with age to peak values after weaning, suggesting that kidney G6Pase may have a different metabolic role. *In situ* hybridization analysis demonstrated that intestine as well as liver and kidney express the *G6Pase* gene. In contrast to hepatic *G6Pase* gene expression, G6P uptake by liver microsomes is not detectable in the embryo but appears following birth, increasing with age to maximal activity at about four weeks of age. The marked difference in hepatic microsomal G6P transport activity between neonatal and adult mice raises the possibility that differential screening may allow the cloning and characterization of this gene.

The *G6Pase* gene is expressed in a tissue-specific manner in the liver and kidney. To understand the molecular mechanisms regulating liver-specific expression of the *G6Pase* gene, we characterized *G6Pase* promoter activity by transient expression assays. The *G6Pase* promoter is active in HepG2 hepatoma cells, but inactive in JEG3 choriocarcinoma or 3T3 cells. DNA elements essential for optimal and liver-specific expression of the *G6Pase* gene are contained within nucleotides -234 to +3. Deletion analysis revealed that the *G6Pase* promoter contains three activation elements (AEs) at nucleotides -234 to -212 (AE-I), -146 to -125 (AE-II), and -124 to -71 (AE-III). AE-I contains binding sites for hepatocyte nuclear factors (HNF) 1 and 4. Electrophoretic mobility shift and co-transfection assays demonstrated that HNF1α, but not HNF4, binds to its cognate site and transactivates *G6Pase* gene expression. The *G6Pase* promoter contains five HNF3 motifs: HNF3 site 1(-180/-174); HNF3 site 2 (-139/-133); HNF3 site 3 (-91/-85); HNF3 site 4 (-81/-75); and HNF3 site 5 (-72/-66). All five sites bind to HNF3γ with high affinity. Transient expression and co-transfection assays showed that HNF3 site 1 is not required for basal promoter activity, but is essential for HNF3γ-activated transcription from the *G6Pase* promoter. We further showed that HNF3 sites 3, 4, and 5 are essential for basal *G6Pase* promoter activity and transactivation by HNF3γ. AE-II contains, in addition to a HNF3 motif, a cAMP-response element (CRE) and a C/EBP half-site. The *G6Pase*(-146/-116) DNA containing AE-II forms multiple protein-DNA complexes with HepG2 nuclear extracts, including HNF3γ, CRE-binding protein (CREB), C/EBPα, and C/EBPβ. We showed that AE-II mediates transcription activation of the *G6Pase* gene by cAMP.

Methionine Adenosyltransferase I/III Deficiency. Methionine adenosyltransferase (MAT) is a key enzyme in transmethylation, in transsulfuration, and in the biosynthesis of polyamines. We have shown that MAT I/III deficiency, characterized by isolated persistent hypermethioninemia, is caused by mutations in the *MAT1A* gene encoding MATα1, the subunit of major hepatic enzymes, MAT I [$(\alpha1)_4$] and III [$(\alpha1)_2$]. We have characterized ten *MAT1A* mutations in MAT I/III deficient individuals and shown that the associated hypermethioninemic phenotype is inherited as an autosomal recessive trait. However, dominant inheritance of hypermethioninemia, also thought to be caused by MAT I/III deficiency, has been reported in two families. We have now shown that the only mutation uncovered in both families is a G→A transition at nucleotide 791 in exon VII of one *MAT1A* allele, which converts an arginine at position 264 to a histidine (R264H). Substitution of Arg-264 with histidine (R264H, the naturally occurring mutant), leucine (R264L), aspartic acid (R264D), or glutamic acid (R264E) greatly reduces MAT activity and severely impairs the ability of the MATα1 subunits to form homodimers essential for optimal catalytic activity. On the other hand, when lysine is substituted for Arg-264 (R264K), the mutant α1 subunit is able to form dimers that retain significant MAT activity, suggesting that amino acid 264 is involved in inter-subunit salt bridge formation. Cotransfection studies show that R264/R264H MATα1 heterodimers are enzymatically inactive, providing an explanation for the R264H-mediated dominant inheritance of hypermethioninemia.

HERITABLE DISORDERS OF PHOSPHOLIPID AND LIPID-MODIFIED PROTEIN METABOLISM

The Section on Developmental Genetics under ***Anil Mukherjee*** conducts research on heritable disorders of metabolism of lipids (e.g., phospholipids) and catabolism of lipid modified (e.g., palmitoylated) proteins. More specifically, the major emphasis of this Section has been to investigate the biochemistry and molecular biology of three genes related to lipid metabolism and/or catabolism of lipid-modified proteins. These genes are: group-I (pancreatic) soluble phospholipase A_2 (*sPLA$_2$-I*) and its inhibitor, uteroglobin (*UG*), and protein palmitoyl thioesterase. Collaboratively, this group also studies the osteopontin (*OPN*) gene, as it relates to complex human genetic diseases such as atherosclerosis.

Role of Lipids in Cellular Functions. During the past decade, compelling evidence has been obtained to suggest that lipids play important roles in many vital cellular functions. Not only are they a critical constituent of the cell membrane, metabolism of some lipids (e.g., phospholipids) is the major source of potent mediators of inflammation, while others are involved in posttranslational modification of proteins that play vital roles in neurotransmission (e.g., anchorage of acetylcholinesterase to the cell membrane), assembly of proteins in the retinal rods and its turnover and in cellular signal transduction (e.g., *HaRas*). In addition, enzymes such as the PLA_2s have been implicated in long-term potentiation and immunological activation. PLA_2s are the key enzymes in the production of arachidonic acid from cellular phospholipids, and play a pivotal regulatory role in the production of eicosanoids (e.g., prostaglandins, leukotrienes and thromboxanes), which are well known mediators of inflammation and cellular differentiation. Soluble group I PLA_2s (sPLA$_2$-I), in addition to their catalytic function, also have a receptor-mediated effect that includes smooth muscle contraction, cell proliferation, invasion of the extracellular matrix, and induction of cyclooxygenase-2 (COX-2). COX enzymes catalyze the metabolism of AA to prostaglandins (Pgs), which are multifunctional lipid mediators of such processes as inflammation and cellular differentiation. While *COX*-1 is constitutively expressed in nearly all tissues, *COX*-2 is inducible, and secretory PLA_2 transcriptionally activates COX-2 gene. This enzyme has been recently reported to be involved in the pathogenesis of inherited gastrointestinal cancers, a role that is supported by the results of epidemiological studies that showed that chronic consumption of aspirin and aspirin-like durgs (inhibitors of COX-2) reduces colorectal cancer mortality by 40-50%. Since PLA_2 is reported to be activated by dietary lipids and colorectal cancer tissues have high PLA_2 activity, it is hypothesized that the PLA_2 gene may play a modifier role in this familial cancer syndrome. Since inflammatory diseases as well as cancers are some of the most important causes of human mortality and morbidity, understanding the regulation (i.e., activation and inhibition) of this enzyme is of great physiological and medical importance.

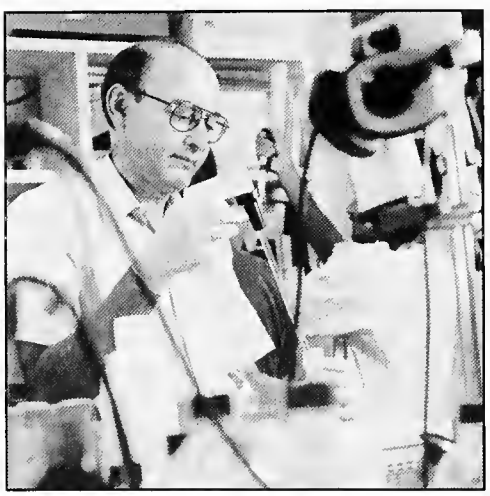
Lipid Modification of Proteins. Like the metabolism of phospholipids, catabolism of lipid-modified proteins is also an extremely important physiological process. This is generally accomplished by enzymatic catalysis, and mutations in the gene encoding at least one these enzymes, protein palmitoyl thioesterase (*PPT*), have been shown to cause infantile ceroid lipofuscinosis (INCL), one of the five subgroups of a relatively common (1:12,500) heritable neurodegenerative disorder, known as neuronal ceroid lipofuscinosis (NCL) or Batten's disease.

Regulation of Phospholipase A2. We have pioneered the study of a family of endogenous proteins, UGs, that inhibit the activity of soluble PLA_2s (sPLA$_2$s) and which therefore have immunomodulatory/antiinflammatory properties. These proteins are not only important from the standpoint of their PLA_2-inhibitory, antiinflammatory activities, but also because the genes encoding these proteins contain very strong promoters. These promoters are of significant interest for gene therapy and targeting of a particular gene to a specific organ (e.g., the lung). Most importantly, UG appears to play critical roles in maintaining normal glomerular function, as targeted disruption of the *UG* gene in mice causes severe glomerulonephropathy, akin to a familial human renal disease reported within the last two years.

More recently, we have discovered that there is a cell surface receptor for UG, and that, using this receptor-mediated pathway, UG regulates such important cellular functions as migration and invasion of the extracellular matrix (ECM). This is a very significant finding, because cell migration is involved in several important biological processes such as inflammation, embryogenesis, and metastasis of cancer cells to distant organs. Cloning and characterization of the cDNA and the gene encoding this receptor protein is expected to facilitate the development of receptor-agonists and antagonists with potential medical applications.

Uteroglobin Knockout Mouse: a Model for Inherited Human Nephropathies. One of our most significant achievements of the last year was the discovery of the physiological function of UG. By targeted disruption of the murine *UG* gene in embryonic stem (ES) cells, we found that animals lacking UG develop a severe fibronectin (Fn)-deposit glomerulonephropathy. This is due to the fact that UG binds to Fn with high affinity and prevents self-aggregation and multimerization of Fn, essential for abnormal cellular deposition. Although Fn concentration in human blood is relatively high, we do not understand how abnormal cellular deposition of Fn is prevented. Fn is one of the major constituents of the ECM, and its abnormal accumulation has been reported in atherosclerosis and inflammatory glomerulonephropathies. Since Fn self-aggregation is essential for its abnormal cellular deposition, our results demonstrating the presence of a UG-Fn heteromer in the plasma may, at least in part, explain why circulating Fn is not normally deposited in different tissues and organs. More importantly, the striking similarities in histopathological findings as well as in clinical presentation and disease course between a recently reported familial Fn-deposit glomerulonephropathy and the phenotype of the UG-deficient mice may suggest a common molecular mechanism of pathogenesis. In a collaborative study, we are now trying to determine whether the human disease is due to mutations in the *UG* gene or in its receptor gene.

Novel Receptor-Mediated Function of Uteroglobin. In a related study, we have demonstrated that the putative UG-receptor is expressed on normal trophoblasts, renal mesangia, mastocytoma, fibroma, and lymphoma but not on fibrosarcoma or choriocarcinoma cells. UG appears to regulate cell motility and invasion of the extracellular matrix by cells that express this putative receptor. The expression of the UG-binding protein appears to be stimulated by treatment of the cells with lipopolysaccharide (LPS) and IL-6. Thus, differential display using RNA from cells treated and untreated with LPS and IL-6 provides a unique opportunity for cloning the cDNA encoding the receptor. We have also purified this binding protein by UG-affinity chromatography. Currently, its characterization, including an N-terminal sequence analysis, is under way.


Anil Mukherjee

Because of the emergence of a high-affinity, cell surface receptor for UG, it is important to determine the quaternary structure of this protein. Last year, we reported resolution of the crystal structure at 3.1 Å resolution. Since then, we have sought to determine the solution structure of recombinant human UG (hUG) by multidimensional NMR as well as by crystallography at 2.5 Å resolution. The results show that hUG is virtually identical to that of the rat and rabbit, thus establishing the fact that this is a structurally highly conserved protein. We have also cloned and characterized the *hUG* gene, which is nearly identical in organization to that of the mouse, rat, and rabbit. We found that the *hUG* gene is localized on chromosome 11q*12.3-13.1*, a region that harbors many candidate disease genes.

Novel Receptor-Mediated Function of Uteroglobin. Soluble PLA_2 ($sPLA_2$), like its inhibitor, UG, also has a cell surface receptor, the cDNA and gene for which have been cloned and characterized. We have demonstrated that, via its high-affinity receptor, pancreatic $sPLA_2$ dramatically increases the motility and ECM invasion by NIH 3T3 cells *in vitro*. In a related study, we also demonstrated that transcriptional regulation of $sPLA_2$-induced, receptor-mediated induction of COX-2 is achieved via nuclear factor (NF)-IL-6. This is a very significant finding, because the transcriptional regulation of expression of COX-2 may broaden our understanding of the mechanism(s) of pathogenesis of familial colorectal cancers and facilitate the development of novel therapies. Within the last year, we have also cloned and characterized the cDNA and the gene encoding murine *$sPLA_2$*-I. It is now possible to generate both transgenic mice overexpressing this enzyme, as well as mice carrying a null mutation in the *$sPLA_2$*-I gene, by targeted disruption of the gene in ES cells. Both these animal models are presently being developed.

Infantile Ceroid Lipofuscinosis and Protein Palmitoyl Thioesterase Gene. As indicated above, lipid modification of proteins plays important physiological roles. One of these posttranslational modifications, palmitoylation, is essential for many vital cellular functions and is a reversible process. Palmitoylated proteins are depalmitoylated by protein palmitoyl thioesterase (PPT), and then degraded by lysosomal hydrolases. It has been reported that mutations in the gene encoding PPT cause inactivation of this enzyme and are the genetic defect

in infantile neuronal lipofuscinosis (INCL), for which there is no effective therapy. In order to address the physiological roles of PPT and to generate an animal model for INCL, we have cloned and characterized a murine cDNA encoding this enzyme. Using this cDNA as the probe we have also obtained two genomic clones which, when characterized, will be used to generate *PPT*-null animals. If successful, this animal model of INCL will allow the testing of novel treatments, including gene therapy.

DYSMORPHIC SYNDROMES

The Unit on Molecular Dysmorphology, led by ***Forbes Porter***, is interested in studying the molecular and biochemical processes that underlie birth defects and dysmorphic syndromes. Initial work has focused on defining the role of two related LIM homeobox genes, *Lhx2* and *Lhx7*, in development. Gene targeting in embryonic stem cells has generated mice lacking functional Lhx2. *Lhx2* mutant mice are anophthalmic, have forebrain anomalies, and die *in utero* due to failure of definitive erythropoiesis. Using these mice, and differential display PCR techniques, we have identified twenty three potential down-stream target genes of *Lhx2*. We are currently characterizing these genes further. By defining the genetic hierarchy in which Lhx2 functions, we hope to gain insight into the molecular processes that underlie these malformations. In collaboration with the LMGD, we are producing a mouse in which *Lhx7*, a LIM homeobox gene closely related to *Lhx2*, has been inactivated by gene targeting. Future experiments will allow us to learn how these two genes, independently and in combination, function during development.

Smith-Lemli-Opitz Syndrome. In a line of investigation designed to complement the use of mouse models to study the molecular basis of dysmorphology, we are starting a clinical protocol to evaluate children afflicted with Smith-Lemli-Opitz syndrome (SLOS). These children have abnormal facial features, limb defects, genital anomalies, and brain malformations. Smith-Lemli-Opitz syndrome is likely to be due to a defect in cholesterol biosynthesis. Children with this disorder are currently being treated with dietary cholesterol supplementation. This clinical protocol will focus on the neurological and endocrinological aspect of this disease. Using a magnetic resonance imaging technique called Magnetic Transfer Imaging, we plan to evaluate the myelination state of the brain in children with SLOS. We are interested in determining whether dietary cholesterol supplementation affects myelination in the brain or whether alternative therapies need to be considered. A basic laboratory effort will support these clinical studies. We are currently trying to isolate the gene mutated in this syndrome. The goal is to establish a genotype/phenotype correlation for this syndrome and to produce a genetic mouse model of this human syndrome. By studying the biochemical and molecular perturbations in this syndrome, we hope to better understand the developmental defects it causes and to gain knowledge that can be applied clinically to help these patients and their families.

Forbes Porter

HERITABLE DISORDERS BRANCH

Chief
Joan C. Marini, M.D., Ph.D.

Principal Investigators
Janice Y. Chou, Ph.D.
William A. Gahl, M.D., Ph.D.
Anil B. Mukherjee, M.D., Ph.D.
Ida S. Owens, Ph.D.

Tenure-Track Investigator
Forbes D. Porter, M.D., Ph.D.

Scientist Emeritus
James B. Sidbury, M.D.

Senior Fellows
David Chen, Ph.D.
Marco Ciotti, M.D.
Qi Hui Gong, Ph.D.
Mary McMaster, M.D.

James Pash, Ph.D.
Vorasuk Shotelersuk, M.D.

Guest Researchers
Yair Anikster, M.D.
Lawrence Charnas, M.D., Ph.D.
Emma Jean deBrohun Butler, Ph.D.
Ramasubbar Dhanireddy, M.D.
Lynn Duffy, M.D.
Ozlem Goker-Alpan, M.D.
Juanru Guo, M.D.
Patricia Hartz, Ph.D.
Senator Hazelwood
Rika Hiraowa, M.D.
Gordon Holt, Ph.D.
Donna Krasnewich, M.D., Ph.D.
Sondra Levin, M.D.
Brian Mansfield, Ph.D.
Aprile Pilon-Clayton, Ph.D.
Robert Pretzlaff, M.D.
Chun Zhao, M.D., Ph.D.

Post-Doctoral Fellows
Borhane Annabi, Ph.D.
Nikhil Basu, Ph.D.
Margaret Chamberlin, Ph.D.
Paul Dawson, Ph.D.
Antonella Forlino, Ph.D.
Hisayuki Hiraiwa, M.D.
Stivelia Kachilele, Ph.D.
Gopal Kundu, Ph.D.
Felicitas Lacbawan, M.D.
Dietrich Machleder, Ph.D.
Asim Mandal, Ph.D.
Geraldine McDowell, Ph.D.
Chiun-Jye Yuan, Ph.D.
Zhongjian Zhang, M.D., Ph.D.
Adriana Zingone, M.D.

Pre-Doctoral Fellows
Laura Green
David P. Larson
Raili Seppala-Lehto

Senior Technical Specialists
Isa Bernardini
Chi-Jiunn Pan

ANNUAL BIBLIOGRAPHY

Carlomagno T, Mantile G, Bazzo R, Miele L, Paolillo L, Mukherjee AB, Barbato G. Resonance assignment and secondary structure determination and stability of the recombinant human uteroglobin with heteronuclear multidimensional NMR. J Biomol NMR 1997;9:35-46.

Chamberlin ME, Ubagai T, Mudd SH, Levy HL, Chou JY. Dominant inheritance of isolated hypermethioninemia is associated with a mutation in the human methionine adenosyltransferase 1A gene. Am J Hum Genet 1997;60:540-6.

Chen D, Guo J, Gahl WA. Rab GTPases expressed in human melanoma cells. Biochim Biophys Act 1997;1355:1-6.

Chen D, Guo J, Miki T, Tachibana M, Gahl WA. Molecular cloning and characterization of rab27a and rab27b, novel human rab proteins shared by melanocytes and platelets. Biochem Mol Med 1997;60:27-37.

Ciotti M, Marrone A, Potter C, Owens IS. Genetic polymorphism in the human UGT1A6 (Planar Phenol) UDP-glucuronosyltransferase: pharmacological implications. Pharmacogenetics 1997;7:485-95.

Ciotti M, Obrary R, Martin MG, Owens IS. Genetic defects at the *UGT1* locus associated with Crigler-Najjar Type I disease: including a prenatal diagnosis. Amer J Med Genetics 1997;68:173-8.

Forlino A, D'Amato E, Vallie M, Camera G, Hopkins E, Marini JC, Cetta G, Coviello DA. Phenotypic comparison of an Osteogenesis Imperfecta type IV proband with a de novo α2(1) GLY922→SER substitution in type I collagen and an unrelated patient with an identical mutation. Biochem and Mol Med 1997;62::26-35.

Gahl WA. Carbohydrate-deficient glycoprotein syndrome: hidden treasures (editorial). J Lab CLin Med 1997;129:394-5.

Gahl WA. Nephropathic cystinosis. Pediatr Rev 1997;18:302-4.

Gahl WA, Krasnewich DM, WIlliams JCI Sialidoses. In: Vinken PJ, Bruyn GW, eds. Handbook of clinical neurology. Amsterdam: Elsevier, vol. 66. 1996;353-75.

Gahl WA, McDowell G, Kaiser-Kupfer MI. Benign cystinosis patients [letter]. Cornea 1996;15:101-2.

Gnarra JR, Ward JM, Porter FD, Wagner JR, Devor DE, Grinberg A, Emmert-Buck MR, Westphal H, Klausner RD, Linehan WM. Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. Proc Natl Acad Sci USA 1997;94:9102-7.

Good DJ, Porter FD, Westphal H, and Kirsch IR. Deletion of NHLH-2, a basic helix-loop-helix transcription factor, results in hypogonadism. Nat Genet 1997;15:397-401.

Grassi G, Forlino A, Marini JC. Cleavage of collagen RNA transcripts by hammerhead ribozymes *in vitro* is mutation-specific and shows competitive binding effects. Nucleic Acids Res 1997;25:3451-8

Grassi G, Marini JC. Ribozymes: structure, function, and potential therapy for dominant genetic disorders. Ann Med 1996;28:499-510.

Hazelwood S, Shotelersuk V, Wildenberg SC, Chen D, Iwata F, Kaiser-Kupfer MI, White JG, King RA, Gahl WA. Evidence for locus heterogeneity in Puerto Ricans with Hermansky-Pudlak syndrome. Am J Hum Genet 1997;61:1088-94.

King CD, Green MD, Rios GR, Coffman BL, Owens IS, Bishop WP, Tephly TR. The glucuronidation of exogenous and endogenous compounds by stably expressed rat and human UDP-glucuronosyltransferase Arch Biophys 1996;332:92-100.

Kotb M, Mudd SH, Mato JM, Geller AM, Kredich NM, Chou JY, Cantoni GL. Consensus nomenclature for the mammalian methionine adenosyltransferase genes and gene products. Trends Genet 1997;13:51-2.

Krasnewich D, Gahl WA. Carbohydrate-deficient glycoprotein Syndrome. Adv Pediatr 1997;44:109-40.

Kundu GC, Mukherjee AB. Evidence that porcine pancreatic phospholipase A_2 via its high-affinity receptor stimulates extracellular matrix invasion by normal and cancer cells. J Biol Chem 1997;272:2346-53.

Lin B, Morris DW, Chou JY. The role of HNF1α, HNF3γ, and cyclic AMP in glucose-6-phosphatase gene activation. Biochemistry 1997;36:14096-106.

Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Iyanagi T, Lancet D, Louisot P, Magdalou J, Roy Chodhury J, Tephly TR, Tipton KF, Nebert DW. The UDP-glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenetics 1997;7:255-69.

Marini JC, Gerber NL. Osteogenesis imperfecta rehabilitation and prospects for gene therapy. JAMA 1997;277:746-50.

McDowell GA, Gahl WA. Inherited disorders of glycoprotein synthesis: cell biological insights. Proc Soc Exp Biol Med 1997;215:145-57.

Miele L, Cordella-Miele E, Xing M, Frizzell R, Mukherjee AB. Cystic fibrosis gene mutation (deltaF508) is associated with an intrinsic abnormality in Ca2+-induced arachidonic acid release by epithelial cells. DNA Cell Biol 1997;16:749-59.

Owens IS, Ritter JK, Yeatman MT, Chen F. The novel *UGT1* gene complex links bilirubin, xenobiotics, and therapeutic drug metabolism by encoding UDP-glucuronosyltransferase isozymes with a common carboxyl terminus. J Pharmacokinet Biopharm 1996;24:491-508.

Panda D, Kundu GK, Lee BI, Peri A, Fohl D, Chackalaprampil I, Mukherjee BB, Senger D, Li XD, Mukherjee DC, Seides S, Rosenberg J, Stark K, Mukherjee AB. Potential roles of osteopontin and $\alpha_v\beta_3$ integrin in coronary restenosis. Proc Natl Acad Sci USA 1997;94: 9308-13.

Parvari R, Lei, K-J, Bashan, N, Hershkovitz E, Korman SH, Barasj V, Lerman-Sagie T, Mandel H, Chou JY, Moses S. Glycogen storage disease type 1a in Israel: biochemical, clinical, and mutational studies. Am J Med Genet 1997;72:286-90.

Parvari R, Lei, K-J, Szonyi L, Narkisl G, Moses S, Chou JY. Two new mutations in the glucose-6-phosphatase gene cause glycogen storage disease in Hungarian patients. Euro J Hum Genet 1997;5:191-5.

Porter FD, Drago J, Xu Y, Cheema SS, Wassif C, Huang SP, Lee E, Grinberg A, Massalas JS, Bodine D, Alt F, Westphal H. *Lhx2*, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. Development 1997;124:2935-44.

Shanmugam V, Chackalaparampil I, Kundu GC, Mukherjee AB, Mukherjee BB. Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells. Biochemistry 1997;36:5729-38.

Zhang Z, Kundu GC, Yuan C-Y, Ward JM, Lee EJ, De Mayo F, Westphal H, Mukherjee AB. Severe fibronectin-deposit renal glomerular disease in mice lacking uteroglobin. Science 1997;276:1408-12.

Zhang Z, Zimonjic DB, Popescu NC, Wang N, Gerhard D, Stone E, Arbour NC, de Vries MA, Scheffer H, Garritsen J, Colle'e J, ten Kate LP, Mukherjee AB. Human uterogobin gene: structure, subchromosomal localization, and polymorphism. DNA Cell Biol 1997;16:73-83.

LABORATORY OF CELLULAR AND MOLECULAR BIOPHYSICS

Joshua Zimmerberg, M.D., Ph.D., Chief

This Laboratory aims to elucidate the physical basis of biological phenomena, to provide a foundation for understanding physiological and pathophysiological mechanisms. To this end, the Laboratory develops and uses novel, non-invasive technologies to probe physical parameters of living systems and their components. Our investigators also study systems of well-defined molecular composition and structure that exhibit an essential biological function. This approach provides an environment that supports investigation of the physico-chemical basis of molecular, physiological, and pathological processes. Research in the Laboratory includes the biophysics of gas phase organic ions and peptides, polymer organic chemistry for separation of biological macromolecules and particles, membrane biophysics and electrophysiology of ion channels and membrane merger, cellular biophysics of parasite entry and viral infection, and the cellular and tissue basis of human lymphoid tissue function and HIV pathogenesis therein.

MEMBRANE REMODELING DURING VIRALLY INDUCED SYNCYTIA FORMATION

In the Section on Membrane and Cellular Biophysics, directed by ***Joshua Zimmerberg***, we study electrophysiological cell surface changes during virally induced pathological events. Membrane topology defines life: the inside of a cell is highly organized at the molecular level, at almost solid-state densities; the outside environment of cells is usually more chaotic. To understand the mechanisms by which cells control their membranes' topologies, we have continued to develop new methods and to use new and old techniques to study aspects of cell membrane remodeling. The fusion pore is the object through which viruses infect cells. It is essential that we understand this structure at the highest level of resolution in order to design ways of stopping viral infection at the stage of cell entry. These studies are allowing us to manipulate proteins and membranes in order to determine which dominates fusion pore characteristics. In addition, the detailed mechanistic pathway of membrane fusion is not known in any system, although there are myriad examples of membrane fusion in normal human development, starting with gamete interactions and continuing throughout life. Viral infection and granuloma formation are only two examples of pathological membrane fusion. It is likely that all these membrane fusion events use the same mechanistic pathway as does viral syncytia formation.

By studying the biophysical mechanisms of protein-mediated membrane fusion, we hope to learn how the process of membrane fusion is catalyzed and regulated by biological organisms in health and in disease states. The best known protein that catalyzes fusion is the hemagglutinin of *influenza* (HA). We have developed a system to study membrane fusion at the molecular and physico-chemical level, using simultaneous electrical and fluorescent signals. This system requires in-house programming of computer interfaces to record these small signals. This year, we concentrated on understanding the relationship between hemifusion and complete fusion in the fusion of red blood cells to cells expressing both wild-type and mutant HA molecules.

Fusion Pore Formation and Hemifusion. We studied the interaction of cells expressing the ectodomain of HA linked to a lipid (GPI-HA, BHA-PI cells) with red blood cells (RBC), using time-resolved admittance recordings combined with fluorescence microscopy. RBCs were loaded with membrane marker (PKH26) and bound to BHA-PI cells. As expected, GPI-HA at pH 4.9 causes hemifusion of BHA-PI cell to RBC. One may hypothesize that the ectodomain of wild-type HA (WT-HA), like GPI-HA, also mediates hemifusion, but due to the fast opening of a fusion pore, a transient hemifusion is not detected as a distinct stage. To dissect the distinct stages of fusion, we decreased the efficiency of fusion mediated by wild-type HA. Since fusion pores may be too small to allow the passage of aqueous dyes, we complemented fluorescence microscopy with measurements of the ionic conductances of the pores to determine whether cells hemifuse or harbor small pores permeable to ions. At pH 5.3, the spectrum of response is significantly different from that at pH 4.9. We recorded notable lipid dye redistribution with no pores of conductance (resolution limit: 50-100 pS). When fusion pores are detected, they follow one of the two patterns: (1) pores that open in a similar manner to those described above but usually fail to dilate; and (2) pores that open and close reversibly (flicker) for a short time and then close completely while dye transfer continues ("transient" pores).

We thought it possible that the active conformation of HA at pH 5.3 is different from that at pH 4.9. To experimentally manipulate only the number of activated HA molecules in their optimal conformation (pH 4.9), we took advantage of the fact that the HA0 precursor delivered to the cell surface has to be cleaved into its fusion-competent HA1-S-S-HA2 form by trypsin treatment prior to low pH application. As we reduced the number of fusion-competent HA molecules by shortening trypsin treatments, more HAb2 cells were stained by membrane dye but not by aqueous dye after application of pH 4.9 medium. In simultaneous admittance and fluorescence measurements, lipid dye transfers without any detectable fusion pore formation, which is evidence of hemifusion. Thus, decreasing the density of activated HA molecules by lowering the number of fusion-competent HA molecules, or by lessening the acidity of the activating solution, yields hemifusion. In these experiments with mild trypsin treatment, it is also possible that varying amounts of the uncleaved precursor HA0 remain in the contact site and that this somehow affects fusion. To exclude this possibility, we used a second approach to slow fusion at optimal pH by lowering temperature. With decreasing temperature, an increasing percentage of cells exhibits lipid dye exchange but not aqueous dye exchange, consistent with hemifusion between cells. At 22°C, the majority of fusion pores fail to enlarge or enlarge slowly, attaining conductances of 0.29 to 3 nS at 200 s after opening. Lipid exchange was detected after or in the absence of pore widening.

In agreement with the hypothesis that hemifusion is an intermediate in complete fusion mediated by WT-HA, we found that WT-HA has hemifusion activity. However, to our surprise, the resulting hemifusion state is rather stable. While WT-HA does have an intact transmembrane domain, we never saw an expanding fusion pore, in any of these experiments with WT-HA, after the onset of lipid mixing, although the flickering pores which close completely rather than expanding may precede the stable hemifusion state. These data suggest that the hemifusion state is irreversible. To test the reversibility of hemifusion, we returned populations of cells from 20°C to 33°C after 10 minutes incubation in the low pH medium. No increase in the number of cell pairs with transfer of aqueous dye was observed. Thus, once hemifusion has occurred with WT-HA, it is irreversible.

Joshua Zimmerberg

The absence of fusion pore formation in the presence of the native transmembrane domain and the presence of fusion pore formation for the GPI-HA-mediated fusion suggest that fusion pore formation is not directly caused by the transmembrane domain. The fusion pore is thought to be formed by several activated HA trimers acting in a co-operative manner and assembled in a multiprotein complex. Interacting HA molecules can surround the future fusion site, and start fusion with a local hemifusion. The very same HA molecules may restrict the mobility of lipids (including our lipid dye) and, thus, prevent expansion of the hemifusion intermediate beyond this proteinaceous "fence". This fence would keep tension focused within the fusion site to allow the transformation of local hemifusion into an expanding fusion pore. If the number of activated HA molecules is not high enough to form a sufficiently large and tight complex (less fusion-competent HA, higher pH, or lower mobility of HA), or if the interactions between HA molecules in the complex are weaker (as in the case of GPI-HA 4), the growing hemifusion intermediate can break the HA fence and expand, prior to the opening and expansion of a fusion pore. This would correspond to the detected irreversible hemifusion. Because local merger of membrane lipid bilayers would occur prior to the opening of a fusion pore, this model also explains earlier data on the lipid-sensitive stage of fusion prior to pore formation, a stage common to disparate biological fusion processes.

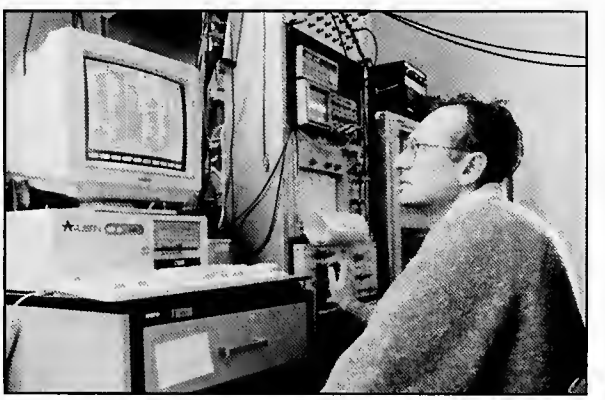
Software for Measuring Membrane Capacitance and Conductance. We have improved our software by porting the real time component to the PC44 supercontroller based upon a digital signal processor, adding new features, improving the user interface, and integrating our software package with standard MS Windows software. The software now traces all modifications of parameters, in a dynamically-linked experimental log. The real time component of the software package has been appended by procedures improving data saving, allowing the user to choose between resetting and preserving the time scale, by a new system of storing data, which allows one to suggest the new data file names and to store data in predefined directories, by display of the time elapsed from the beginning of the experiment with the increment selected by user, by customization of phase increments in manual phase adjustment procedures, by an option to reset time scale or restart data saving in the course of an experiment,

by an enhanced procedure for on-line entering of the compensation parameters, and by an improved system of data plotting fully consistent with the same system for lock-in experiments. Additional procedures include issuing of TTL pulses for synchronization, single and periodic calculations and display of membrane conductance in the course of continuous acquisition, fully automatic phase adjustment based on pulse operated relays for lock-in experiments, and on-line modification of the holding potential in the course of a lock-in experiment.

THE INVOLVEMENT OF LIPIDS IN BIOLOGICAL FUSION INTERMEDIATES

The Unit on Lipid Intermediates in Fusion, led by ***Leonid Chernomordik***, studies how incorporation of different lipids into fusing membranes affect different fusion stages, to further determine the fusion pathway as the sequence of the structural lipid-involving intermediates and to uncover the physical forces that drive rearrangement of two membrane lipid bilayers into one. Our working hypothesis is that the different membrane fusion systems, including fusion of purely lipid bilayers, involve a consecutive formation of two classes of transient and highly bent intermediates: stalks that are lipidic connections between contacting monolayers of fusing membranes; and pores. The stalk-pore hypothesis suggests that the energetics of hemifusion and fusion pore intermediates depends on the spontaneous curvature of different membrane monolayers.

Low Temperature Stabilizes a Restricted Hemifusion Intermediate of Membrane Fusion. Brief exposure of HA-expressing cells with bound RBCs to low pH at 40°C results in establishment of a long-lived fusion-committed state. We found that low temperature reversibly inhibits not only complete fusion but also hemifusion. This low-temperature-arrested stage, LT-S, follows the low pH-dependent conformational change in HA, the insertion of the fusion peptide into the RBC membrane, and the fusion stage arrested by lysophosphatidylcholine (LPC), LPC-S. Our data are consistent with the hypothesis that there is a local lipidic connection between the outer monolayers of fusing membranes at the LT-S: (1) lipid mixing at LT-S, but not at LPC-S, can be promoted by applying osmotic shock, suggesting that low temperature blocks fusion at a stage when membranes are connected; (2) the agent chlorpromazine, which is known to selectively destabilize the hemifusion diaphragm yielding complete fusion when applied to hemifused cells, converts LT-S into complete fusion; (3) if lipid diffusion between membranes is inhibited at LT-S by the circle of activated HA molecules assembled around a fusion site, one may hypothesize that local breakage in this proteinaceous fence would allow some lipid mixing to occur. Indeed, partial cleaving of low pH-forms of HA by very mild treatment with proteinase K results in membrane dye redistribution between fusing cells at LT-S. These results indicate that low temperature arrests fusion at a stage that precedes both lipid mixing and opening of a fusion pore, but which follows HA- and lipid-dependent local hemifusion of membrane monolayers when they are restricted by a fence of activated HA surrounding the fusion site.



Leonid Chernomordik

The Pathway of Membrane Fusion. This restricted hemifusion intermediate spontaneously transforms into complete fusion or stable hemifusion in HA-dependent manner. For the same number of activated HA molecules, fusion inhibition by LPC or low temperature, allowing more time for HA assembly, increases the probability of complete fusion. Our ability to shift cells destined for stable hemifusion to cells that undergo complete fusion merely by increasing the duration of HA interaction indicates that stable hemifusion is an outcome of the same initial pathway as complete fusion. Dissecting the pathway into a series of distinct arrested intermediates should facilitate identification of specific conformation(s) of HA that form and stabilize these fusion intermediates, and that self-assemble into multi-protein complexes around the fusion site. The fact that the lipid dependence and the characteristics of the fusion pore are universal in fusion suggests that the mechanism of protein-driven membrane rearrangements is common for diverse biological fusion reactions.

Alcohol Promotes Hemifusion of Phospholipid Membranes. Recent studies suggest that diverse biological fusion reactions and fusion of protein-free lipid bilayers can all proceed via formation of similar intermediates: stalks, and

local and transient lipidic connections between contacting monolayers of the fusing membranes. While the energy of the existing stalk is mainly determined by the elastic properties of the membrane monolayers, the formation of a stalk requires transient and local breaking of the continuity of the monolayers. To study early stages of lipid bilayer fusion, we modulated the surface properties of lipid bilayers by adding a short-chain alcohol. Methanol, ethanol, and 1,2 propanediol were found to dramatically promote hemifusion between lipid vesicles and planar bilayers. Importantly, short-chain alcohols are known to inhibit the transition between lamellar and inverted hexagonal phases and to thereby support the lipid monolayer curvature that is the opposite to that in a stalk. We hypothesize that incorporation of alcohol onto the bilayer surface decreases its energy and facilitates local and short-lived breaking of the surface, which exposes a hydrophobic part of the bilayer, required for stalk formation, to the aqueous milieu.

COMPONENTS AND KINETICS IN EXOCYTOSIS

A second group, led by ***Joshua Zimmerberg***, studies molecular mechanisms involved in secretion, fertilization, and neurotransmission, to assign functions to proteins and lipids involved in membrane fusion by studying the temporal dependence and properties of identifiable stages in calcium-triggered cortical granule exocytosis. The cortical granule (CG) is well characterized, and can be prepared with high-purity and high-yield preparation for the study of docking and fusion events. Isolated CGs retain their Ca^{2+}-sensitivity for fusion, either to one another, to isolated plasma membrane patches, and even to pure phospholipid vesicles, suggesting that the isolated granules carry with them all the molecular machinery necessary for docking, Ca^{2+}-sensing, and membrane-membrane fusion. The significance of the kinetic study lies in the fact that it allowed us to develop a kinetic model that describes many of the features of calcium-triggered exocytosis, and that this model can be used to evaluate alterations in the behavior of the system as a result of experimental manipulations. This is important because a theoretical model that can explain the complex biological behavior observed during triggered exocytosis can be used to evaluate the role of proteins and lipids in the steps that define the exocytotic process. The biochemical research:(1) further proves that the sea urchin model system is comparable to all other secretory model systems, even at the level of proteins employed in the secretory pathway; (2) shows the extent to which this system is amenable to a reductionist research approach, which cannot be sustained by other model systems, thus limiting the hypotheses that can actually be tested; (3) proves that the SNARE hypothesis must be modified as the membrane constituents identified appear to be involved in docking but not membrane fusion per se; and (4) indicates that the membrane constituent(s) essential to fusion have yet to be identified.

Kinetic Analysis of Calcium-Triggered Exocytosis *in Vitro*. The specific objectives of the kinetic study have been: (1) to extend a general, kinetic model that relates the rate and extent of triggered exocytosis to the number, distribution, and efficacy of activated fusion complexes by including an activation time for fusion complexes; (2) to examine the calcium dependencies of the parameters described in this kinetic model; and (3) to examine fusion kinetics in the presence of exogenous lipids known to inhibit and promote membrane fusion. A three-parameter model describes the fusion kinetics. The three parameters are the probability that a calcium-activated fusion complex becomes a committed fusion complex (q), the probability that a committed fusion complex fuses (p), and the average number of committed fusion complexes, ($<n>$). The relative invariance of $p/<n>$ with calcium is consistent with an intrinsic probability to fuse. The overall probability to fuse is dependent on the total number of committed fusion complexes. There is a characteristic probability (q) for all fusion complexes to enter the committed state, following calcium triggering, which is independent of the concentration of calcium. Heterogeneity is in either the number of granules that enter the committed state from the activated state ($<n>$), or the number that are selected to enter the active state (calcium triggering), or both.

Biochemical Analysis of Egg Cortical Granule Membranes. Using immunoblotting for proteins implicated in fusion, we identified homologs of VAMP2, SNAP-25 and Syntaxin1A on CG membranes at 19 kDa, about 30 kDa, and about 40 kDa, respectively. Additional VAMP- and syntaxin-immunoreactive bands at higher molecular weight (about 70 kDa by SDS-PAGE) were detected when samples were not boiled. Dissection of the 70 kDa band confirmed that it contained the three SNARE proteins. This heterotrimeric complex was also identified in sucrose gradients of NP-40-extracted CG protein. Furthermore, immunoprecipitation using either anti-VAMP or anti-Syntaxin antibodies results in the co-elution of all three proteins. VAMP in the complex is insensitive to proteolysis by tetanus toxin in spite of almost complete cleavage of VAMP monomers.

Dissociation of the CG SNARE complex is Ca^{2+}-sensitive. This is the first report of such a mechanism. Both forms of the complex, that found pre-formed on isolated CG in suspension as well as that formed between CGs when centrifuged into contact, fully dissociate at Ca^{2+} concentrations causing maximal CG-CG fusion. This was verified on sucrose gradients, and by the reappearance of VAMP monomers in Tetx-treated CG samples following the addition of Ca^{2+}.

The SNARE complex is not essential to membrane fusion. A Ca^{2+} dose-response curve indicates that fusion has a lower Ca^{2+} sensitivity than does SNARE complex dissociation. In addition, strontium ions and barium ions, which are know to substitute for Ca^{2+} in triggering fusion, support CG fusion, but with only limited dissociation of the SNARE complex. Irreversible temperature-dependent inhibition of Ca^{2+}-triggered fusion has no effect on the SNARE complex response to Ca^{2+}.

SNARE protein monomers are not essential to fusion. Treatment of CGs with Tetx and Botx results in extensive loss of VAMP and SNAP-25 monomers and some loss of Syntaxin monomers but no inhibition of fusion; early experiments with recombinant n-sec1 protein, which specifically binds to Syntaxin and blocks its binding to other proteins, also had no effect on fusion. Inasmuch as the fusion assay utilizes centrifugation to produce CG-CG contact, it allows us to bypass protein requirements for docking and to look directly at the Ca^{2+}-triggered fusion event.

There is no Ca^{2+}-dependent inactivation of fusion machines, whereas near-maximal Ca^{2+} concentrations appear to inhibit docking. Pretreatment of CGs in suspension with a maximal dose of Ca^{2+} results in full dissociation of pre-formed SNARE complexes; dilution of these samples to basal Ca^{2+} levels prior to fusion assays results in data comparable to controls. However, exposure of CGs to Ca^{2+}, Sr^{2+} or Ba^{2+} while they are being brought into contact results in significant loss of Ca^{2+} sensitivity but has little effect on the fusion responses to Sr^{2+} or Ba^{2+}. Since Ca^{2+} is a potent effector of SNARE complex dissociation but Sr^{2+} and Ba^{2+} have little effect on this complex, we interpret these results to indicate a docking role for the complex formed between contacting CGs. The role of the pre-formed complexes on CG membranes is unclear; implications from some experiments might suggest that they act as reserves of SNARE protein monomers that become available when changes in Ca^{2+} levels trigger fusion.

In summary, there are conditions under which fusion occurs in the relative absence of complex dissociation, and under which complex dissociation occurs despite inhibition of fusion. Thus, there is no direct correlation between SNARE complex dissociation and the extent of fusion, as implied by the SNARE hypothesis, and the complex therefore does not appear to be essential to fusion. It would seem that another membrane constituent, possibly a protein other than the identified SNAREs, may be essential for Ca^{2+}-triggered membrane fusion.

THE ISOLATION AND CHARACTERIZATION OF MACROMOLECULAR AND CELLULAR PARTICLES

The Section on Macromolecular Analysis, led by ***Andreas Chrambach***, pursues the application of analytical and preparative electrophoresis to separation problems, and the development of electrophoresis methodology. Present problems which we are attempting to overcome relate to: (1) the need to separate and isolate the building blocks of biological membranes and subcellular-sized particles, the large size of which rules out the use of conventional gel methods; (2) the absence of user-friendly, high yield isolation methods; (3) lack of rationales in the choice of polymer solutions for achieving "size separations"; (4) the difficulty in achieving solubilization for electrophoretic separations in "non-denaturing" detergents, without disrupting complexes required for biological activity. Analytical methods developed in the Section to solve those problems utilize: (1) the physical characterization of the material migrating as a band on the basis of its mobility at various polymer concentrations, which yields the effective size and surface net charge density of the particle, i.e., the parameters by which particles differ predominantly; (2) the choice of optimally resolving polymer conditions in a non-arbitrary way, i.e., on the basis of theoretical models and physical measurements of parameters such as bandwidth and band asymmetry. The preparative work of the Section aims at an extension to subcellular-sized particles of a recent technique employing automated apparatus (HPGE™) by which an elution cup descends onto the band of interest under computer control to allow for electroelution from gels, or volumetric withdrawal from polymer solutions of the band, at a right angle to the orientation of the electrophoretic separation.

Detection and Isolation of Particle Components. The isolation of fusion-competent particles is a prerequisite for determining the macromolecular composition of these particles and the nature of macromolecular complexes in which the biological activities are maintained. The Section has pioneered preparative electrophoretic methods that

use polymer solutions to achieve effective separations due to size- and shape-differences between particles. The methods were applied to three problems. (1) Rat liver microsomes, which are separated into three electrophoretic components with an 80 % yield for each. Under fusion conditions, the three original components were shown to give rise to at least seven different species which remain to be characterized. (2) Sea urchin (*S. purpuratus*) egg cortical granule preparations exhibited 3 electrophoretic components in 0.02 to 0.05% agarose solutions, only two of which migrate in electrophoresis and differ in size. (3) Sea urchin (*L. pictus*) egg cortical and yolk granules were separated and isolated from 1.5% polyvinylpyrrolidone solution with 70% yield. The load capacity per lane of the 8-lane apparatus is 100 ug (30 ul), with about 35 ug of each granule type recovered. However, the detection of unlabeled granules by "fluorescence reduction" (see below) is incompatible with the automated isolation procedure previously elaborated for fluorescent microsomes, is therefore less reproducible and needs to be upgraded with fluorescently labeled granules.

Separation of Apoptotic Cells. Apoptotic human lymphocytes were separated from viable ones by free-flow electrophoresis (FFE), utilizing the increased mobility (surface charge density) of apoptotic cells at pH 8.3. However, incomplete separation by FFE clearly indicates that this large-scale preparative method still requires considerable systematic and quantitative methodological work before it is sufficiently effective and user-friendly to become widely applicable.

Electrophoretic Separations of Membrane-Protein Complexes Solubilized in Non-Denaturing Detergents. While it has been shown that the syntaxin-VAMP-complex migrates as a single component, undissociated by SDS under cold conditions, it appears likely that the biological structure of the "fusion machine" is severely altered by reaction with a strongly dissociating detergent. It appeared of interest, therefore, to investigate the forms in which the four proteins of the complex exist in a medium of "non-denaturing" detergents such as β-octylglucoside (OG) or CHAPS. Sea urchin (*S. purpuratus*) egg cortical granules were solubilized in OG at concentrations up to 3%. The degree of solubilization increases with OG concentration, reaching a maximum at 1%. Polyacrylamide gel electrophoresis in gels containing 1% OG of the solubilized material was conducted at several gel concentrations, and gels were analyzed by Western blotting, using antibodies to the 4 proteins. The method yields the size of the fragment. Relevance of the fragment to fusion is indicated by its immunological properties.

Capillary and Gel Electrophoresis at Extraordinary Polymer Concentrations of β-Amyloid Peptide. The separation of size-isomeric Congo-red reactive and unreactive β-amyloid peptides is a prerequisite for studies aimed at a macromolecular diagnosis of Alzheimer's disease. To separate two conformers of this peptide, one of which is β-sheeted, two unconventional approaches were taken. (1) The first rests on the recent finding made in the Section that highly concentrated uncrosslinked polyacrylamide allows for the detection of single base substitutions in DNA fragments, obtainable otherwise only the presence of denaturants. Four size-isomeric components were detected in the range of 18-22% uncrosslinked polyacrylamide. (2) The second approach utilized a previous demonstration with angiotensin showing that crosslinked polyacrylamide gels could be formed at up to 50% gel concentration. Since β-amyloid peptide is thought to be a small molecule of between one thousand and a few thousand molecular weight, it was subjected to gel electrophoresis in the 20 to 35% concentration range. Only the first approach reveals more than one component; which of the two is β-sheeted is under investigation. Evaluation by Ferguson plot analysis showed that two of the four components are size isomeric.

The Mechanism of Band Spreading. The control and predictability of band spreading are important since resolution of bands in electrophoresis, as in other separation methods, depends on it. To date, the mechanisms by which bands spread as a function of migration time or distance have been attributed to the sum of a multitude of factors (diffusion, temperature, field strength, sample load, viscosity, electroendosmosis, etc.). Our work this year both in capillary and in gel electrophoresis has fundamentally changed that concept. It was found that under conditions that rule out everyone of those factors, band spreading was a linear function of migration distance only. Mathematical modeling shows that the experimental data are best fitted by a model which assumes that starting zone length and the degree of interaction with the matrix (related to mobility, i.e., surface charge density, size and shape) are responsible for that increase in bandwidth with migration distance.

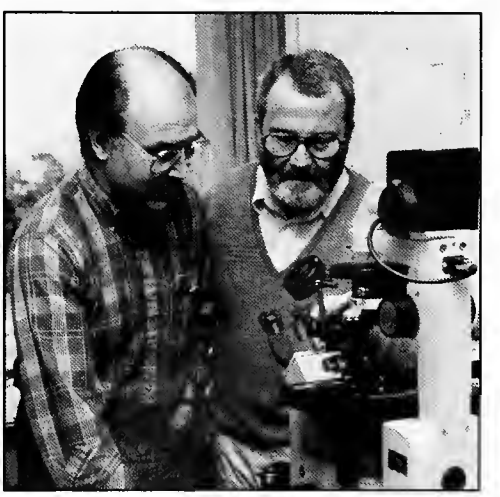
Adaptation of Automated Apparatus to the Separation of Subcellular-Sized Particles. Automated electrophoresis of subcellular-sized particles was advanced in two ways: (1) by automating electroelution from dilute polymer solutions; (2) by increasing the detection sensitivity of "fluorescence reduction". (1) Application of automated horizontal gel apparatus to polymer solutions and large particles was originally conducted with highly concentrated polymer solutions to minimize convection. However, these proved ineffective for size separations. Moreover, when electroelution of macromolecules from gels was replaced by manual volumetric band withdrawal from polymer

solutions, the automation of the preparative method is inevitably lost. This led us to attempt, this year, to separate large particles in dilute (0.09%) solutions of agarose for which resolving capacity had been previously established, and to construct an apparatus modification that allowed us to restore automated band withdrawal. Convective band spreading during electrophoresis of subcellular-sized particles could be minimized at an optimal polymer concentration. The relevant apparatus modification and computer control yielded a preparative technique equivalent to that previously developed for gels. (2) We previously developed a detection technique designated as "fluorescence reduction" for fluorescently unlabeled particles. This technique uses the absorbance of the particle to reduce both the incident light and the emitted fluorescence of a fluorescent paper sheet placed beneath the gel. However, as applied to proteins, this technique yields only 1/30 of the detection sensitivity of fluorescence. To solve that problem, a theory of "fluorescence reduction" was formulated, which shows that increased sensitivity could be achieved by, first, increasing the fluorescence intensity/fluorescent noise ratio of the paper; and, secondly, by increasing the spectral overlap between the analyte and the excitation and emission spectra of the fluorescent paper. The theory also predicted that, within a limited ratio between background fluorescence and band amplitude (of the order of 20%), "fluorescence reduction" yields a linear dose response curve and therefore lends itself to quantitative evaluation. All three predictions of the theory have been experimentally verified.

ENERGETICS OF THE INTERACTION BETWEEN WATER, MEMBRANES, AND MACROMOLECULES

The Section on Mass Spectrometry and Metabolism, led by ***Alfred Yergey***, has continued to apply the knowledge of the physical chemistry of ions in their gas phase to fundamental questions concerning the physical aspects of structural biology: how much energy is required for reactions to proceed in the aqueous medium and how do peptide conformations change as a function of reaction conditions. We measure the energetics of these associations.

Hydration Thermodynamics: Small Water Cluster Measurements. Under equilibrium gas phase ion-molecule reaction conditions, measurements of free energy and enthalpy are comparable to the best values of bulk phase solvation energetics, but are independent of any extra-thermodynamic assumptions. In other words, the equilibrium gas phase measurements potentially represent a better approximation to the thermodynamic ideal value than do the bulk phase measurements. The experimental challenge is to attain equilibrium thermodynamic conditions under circumstances that give adequate ion intensities. The critical aspect of the work is achieving the equilibrium conditions in the "high pressure" ion formation portion of the mass spectrometer while not perturbing ion intensity distributions in the process of making measurements in the low pressure portion of the instrument. The equilibrium states must be obtained in combination with stable conditions for the formation of the ion clusters of interest. In our case, that requires the introduction of the non-volatile organic solutes into the vapor state under well defined conditions of temperature, pressure and solvent concentration. Our experimental approach is to use a reduced pressure electrospray ion source in conjunction with a single stage pressure reduction prior to mass analysis in a cryopumped mass analyzer. This approach has been shown by Kebarle and co-workers to yield equilibrium ion cluster distributions and to reproduce alkali ion delta-Hsolv values obtained under other conditions. We have measured equilibrium constants for several methyl ammonium and anilinium water cluster ions, using the electrospray ionization/equilibrium gas phase/ion-molecule reaction approach described previously by Kebarle. We found satisfactory agreement between our measurements of equilibrium constants of these species and those published previously by Kebarle and others.



Alfred Yergey (left) and Andreas Chrambach

Hydration Thermodynamics: Large Water Cluster Measurements. Under the kinetically limited conditions of ion formation in the high pressure region of an electrospray ionization apparatus, ions are produced surrounded by excess solvent molecules. In analytical applications of electrospray ionization, this excess water is stripped from the analyte ions by a combination of electrostatic acceleration and thermal energy. If one were to reduce electrostatic accelerations in combination with lowered thermal energy input, then ions could be delivered to the

mass analyzer still surrounded by solvent molecules. Ion-water complexes form with a clathrate-like regular dodecahedral structure of hydrogen-bound water molecules. The presence of 20 water molecules, which has been called a "magic number" distribution, is characteristic of some structures.

The energy with which an individual water molecule is bound in such complexes can be measured by carefully controlled physical interactions within the mass spectrometer. Specifically, causing a cluster ion with defined energy and containing some number of solvent molecules, $[M^{+}(H_2O)_n]^{+}$, to collide with a neutral gas molecule, argon for example, results in a fragmentation process, referred to as collision-induced dissociation, CID. The minimum energy at which a CID occurs can be determined. This minimum energy can be related directly to solvation enthalpy. We have used the CID threshold approach to determine energies of tetraethyl and tetramethyl ammonium water cluster ions. Scatter in the threshold determination of the tetramethyl ions is appreciably smaller than for the tetraethyl, due principally to improvement in experimental technique. Nevertheless, we have not yet been able to measure ion intensity values sufficiently close to the onset of bond rupture to place great confidence in the values determined to date. Making the standard correction of the CID values from energy to enthalpy units at 298 K increases the values of the bond energies by about 0.5 kcal/mole, but still clearly places the bond energies for the tetramethylammonium-water cluster bonds below the values that might be expected from those of the methylammonium ions determined by the far more reliable equilibrium method.

Protein/Peptide Conformation Studies. The mass spectra of β-amyloid protein (1-40) associated with neuritic plaques of Alzheimer's disease have unambiguously shown a conformational change in the peptide that is related to neurotoxicity. Electrospray mass spectra of fresh samples of synthetic peptide, βA4, at concentrations ranging from 250 FM to 6 FM, have shown the presence of a mixture of monomeric and aggregated forms of the peptide. Aggregates range in size from dimer to tetramer and are more abundant at the higher concentrations. The most abundant ion of the monomeric form arises from the addition of five protons to the molecule, while the most abundant dimer results from the addition of nine protons. As the original fresh samples are aged, we observe a decrease of about 0.6 for the average charges associated with the monomer. This is interpreted as a loss in charge site availability and is associated with a conformational change in the monomer to a more compact form. At the same time, the aged samples show no loss in average charges associated with the aggregate forms, but exhibit a loss in their intensity relative to the monomers. We interpret these observations as being the consequence of a relatively open conformation present in fresh solution. This open form is capable of forming aggregates in a dynamic equilibrium which leads to reduced aggregation upon dilution. Upon aging, the open monomeric form alters to a more compact form with a loss in average charge and a reduction in the abundance of aggregates of the open form. We have developed a model for the conformational changes of the peptide that is consistent with both the mass spectrometric observations and physical measurements using NMR and CD.

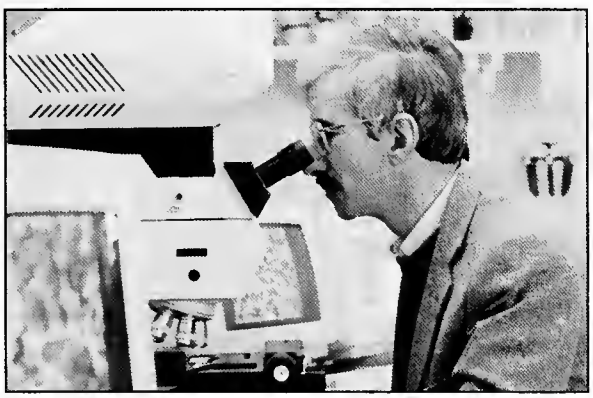
CELL INTERACTIONS IN THREE-DIMENSIONAL TISSUE CULTURE

The Unit on Intercellular Interactions, led by ***Leonid Margolis***, studies the complex cell-cell interactions in tissue, which are the basis for all of the aspects of normal and pathological immune responses *in vivo*. Dysfunction of the human immune system results if interactions of cells in the lymphoid tissue are impaired. The most notable example of such dysfunction is HIV-disease. The ability to study the mechanisms of various immunopathological states under controlled conditions *in vitro* and within the context of human lymphoid tissue is important for answering basic questions on immunopathogenesis, including the dysregulation of the immune response caused by HIV.

HIV-Induced Immunodeficiency in Human Lymphoid Tissue *in Vitro*: Isolate Dependence. We found that cultured tissue blocks of human tonsils, when challenged *in vitro* with tetanus toxoid (TT) or diphtheria toxoid (DT), respond by producing specific antibodies between days six and eight after immunization. Antibody concentration continues to increase up to days 14 to 16 of culture. The response is antigen-specific: challenge by either one of these antigens neither induces the production of antibody against the other antigen, nor increases total IgG production. Infection of human lymphoid tissue with laboratory strains and primary isolates of HIV-1 dysregulates this immune response *in vitro* in an isolate-dependent manner. Infection with a T-tropic HIV-1 strain dramatically suppresses the tissue immune response to TT *in vitro*. In contrast, infection of tissue blocks with the prototypic M-tropic HIV strain enhances the production of specific anti-TT IgG approximately two-fold. Thus, viral tropism is the determinant of humoral immunodeficiency in human lymphoid tissue *in vitro*.

B Cell Dysfunction in HIV-Infected Human Lymphoid Tissue *in Vitro*. A complex combination of soluble cellular factors and T-B cell interactions is required for continuing production of antigen-specific immunoglobulins. To determine whether B cell function is affected indirectly due to an altered microenvironment or directly by the changes in B cells themselves, we isolated lymphocytes from both control and HIV-infected tissues and compared their proliferative response to specific B cell activators. Cells isolated from LAV.04-infected tissue on day 8 to 10 after infection are approximately four-fold less responsive to the T cell-independent B-cell activators than cells from either controls or from equally responsive SF162-infected tissues, regardless of cell purity. Incubation of B cells from non-infected tissue blocks in HIV-conditioned media does not alter their proliferative response to B-cell stimulators. Thus, factors mediating the impairment of intrinsic B cell function seem to be confined to the HIV-infected tissue (e.g., cell-cell contacts, or short-range, rapidly degraded molecules), and are not released into the media in substantial amounts.

Endogenous CC Chemokines in HIV-Infected Human Lymphoid Tissue *in Vitro*. Infection with the T-tropic HIV isolate LAV.04 dramatically increases the amount of CC chemokines produced by lymphoid tissue by day 6 after infection. During days 10 to 12 post-infection, tissue blocks infected with LAV.04 release approximately ten times more MIP-1α and six times more MIP-1β than do control samples (uninfected tissues from the same donor). RANTES secretion also rises, although much less so. The M-tropic isolate SF162 has no effect on endogenous levels of MIP-1α, MIP-1β, or RANTES produced by lymphoid tissue. Thus, paradoxically, MIP-1α and MIP-1β in lymphoid tissue are upregulated by CCR5-independent LAV.04, but are not affected by CCR5-tropic SF162. Although the role of endogenous chemokines in modulating HIV replication in tissue is not known, T-tropic viral induction of CC chemokines may contribute to the shift of viral tropism in HIV-infected patients. T-tropic virus may slow down replication of the dominant M-tropic virus in the course of HIV-1 disease by stimulating production of MIP-1α and MIP-1β, which are inhibitory for M-tropic but have no effect on T-tropic HIV-1. This may provide a competitive advantage for T-tropic HIV-1 variants in the course of HIV-1 infection *in vivo*.



Leonid Margolis

Exogenous CC-Chemokines Inhibit Replication of CCR5-Dependent HIV-1 Variant in Human Lymphoid Tissue *in Vitro*. Replication of the M-tropic variant of HIV-1 SF162 in human tonsillar histocultures incubated with lower concentrations of MIP-1α is highly variable among tissues from different donors. At one extreme, we found that one of the low concentrations of MIP-1α (1 nM or 10 nM) significantly stimulated SF162 replication compared with the control tonsillar tissue from the same donor. In other tissue blocks, there was either no effect on SF162 replication, or it was inhibited by these concentrations of MIP-1β. Variability in suppressor activity at low concentrations of CC chemokine is not the result of donor-to-donor variation in the metabolism or clearance of chemokines, as revealed by comparing the amount of MIP-1α added to the medium with that collected from the cultures.

In contrast to low doses of CC chemokines, replication of SF162 is invariably inhibited by MIP-1α when it is added at a concentration of 100 nM. At day 12 post-infection, p24 production in the MIP-1α-treated histocultures was approximately 8 fold lower than in infected untreated control tissues from the same tonsils. Similar results were obtained in experiments with MIP-1β. MIP-1α (100 nM) also inhibits viral replication in blocks of human mesenteric lymph nodes infected *in vitro* with SF162. The inhibition of HIV-1 replication is virus-specific: MIP-1α at 100 nM failed to inhibit infection with the T-tropic strain LAV.04.

Our results demonstrate that exogenous MIP-1α and MIP-1β inhibit replication of M-tropic but not of T-tropic HIV-1 in human lymphoid tissue. For significant inhibition of viral infection, the continuous presence of high doses of CC chemokines (possibly in combination with other antivirals) is required. The system of cultured blocks of intact human lymphoid tissue may be useful for guiding the development of combinations of CC chemokines or CC chemokine-based drugs with other antivirals for clinical application.

NIH/NASA CENTER FOR TISSUE CULTURE

The Center, funded jointly by NICHD and NASA, featuring a novel Rotating Wall Vessel (RWV) Bioreactor for maintaining and promoting both the original differentiated state of various tissues and the association and differentiation of isolated cells into tissue-like aggregates, continued its activity for the second year. This year, the Center moved into its permanent location in Bldg. 10. Ten research groups used the Center facilities to supplement their main projects with experiments that involve the techniques developed at the center. To evaluate tissue function, the Center is providing a facility for imaging, which includes image processing and single confocal and dual photon scanning laser microscopy. As in the previous year, NIH scientists were invited to submit a short proposal for pilot projects. Bioreactors, supplies, and trained technical support are available to investigate the fate of cells or tissues cultured from one cell type or co-cultures, thin tissue slice culture, or suspended aggregate or tissue culture. Histology and confocal microscopy are provided to evaluate results. Principal investigators must provide direction concerning specific aspects of their project. There is no fee involved in using the Center facilities. On the contrary, principal investigators who are successful in obtaining preliminary data in Phase 1 indicating utility of the Bioreactor for solving important problems are invited to submit a proposal for Phase II studies, which involve funding of a limited number of projects. The following is a list of ongoing projects.

Differentiation of Salivary Cell Culture in the RWV Bioreactor. Hynda Kleinman and M. Hoffmann (NCI) found that the RWV Bioreactor is able to induce acinar differentiation when human submandibular cells are exposed to basement membrane matrix and the laminin-1 protein. In the Bioreactor, smaller amounts of these differentiating substances are required than in conventional tissue culture. Using synthetic peptides, it has been found that one peptide from the G domain of the alpha-1 and alpha-2 chains of the laminin molecule, promotes acinar-like formation. The receptor for this sequence has been found to be syndecan-1, a proteoglycan known to bind to laminin-1. A second peptide sequence within the laminin-1 molecule has recently been identified that is very active with human salivary gland cells maintained in the RWV Bioreactor.

Development of Long-Term Three-Dimensional Cultures of Intestinal Tissue Culture Cells: Interaction of Bacteria and Parasites. Darcy Hanes (Food and Drug Administration, Washington, DC), in collaboration with the staff of the NASA/NIH Center, found that *Cyclospora* parasites adhere to the surface of the CaCo-2 cells differentiated in the Bioreactor for 19 days. By day four post-infection, parasite excystation was observed and sporocyst/sporozoite structures could be seen to emerge from the oocysts. Kinetic studies measuring the number of oocysts associated with the CaCo-2 cells and the number of oocysts in the bioreactor media suggest that a productive infection of the cells with *Cyclospora* occurred. This is the first *in vitro* system described in which this clinically important parasite undergoes its entire life cycle. The ability to culture *Cyclospora sp.* opens the way for both basic studies of this parasite, and for the development of diagnostic tools to identify this organism in humans.

Human Lymphoid Tissue and HHV8, a Model for the Pathogenesis of Kaposi's Sarcoma. Paul Duray (NCI) and Steven Hatfill (NICHD) have co-cultured normal human spleen and tonsillar tissue with BCBL-1 cells, which harbor the HHV8 viral genome. In four out of five experiments, BCBL-1 cells stimulated to undergo lytic HHV8 production were able to generate histological lesions in human spleen and lymph node that were consistent with a clinical pathological diagnosis of Kaposi's Sarcoma. The generation of lesions during these experiments partially fulfills one of Koch's postulates concerning the causes of microbiological-disease.

RWV Bioreactor Models of Human Squamous Metaplasia. Bruce Johnson, NCI, National Naval Medical Center, in collaboration with the staff members of the NASA/NIH Center, have continued their work on the evaluation of retinoic acid for the management of bronchial atypical metaplasia in human patients. In parallel with the clinical studies performed at the Naval Medical Center, the biopsies with a pre-existing bronchial epithelial metaplasia were cultured in the RWV Bioreactor. It was found that the RWV Bioreactor is able to maintain a pre-existing metaplastic epithelial phenotype with gross areas of bronchial metaplasia on bronchoscopy. This metaplasia was confirmed by immunohistochemistry and was maintained for 21 days of the experiment in otherwise histologically normal surrounding human oropharangeal tissue.

Long-Term Maintenance of Human Prostate Tissue in the RWV Bioreactor. Duray (NCI) and Hatfill (NICHD) have utilized the previous experience of the NASA/NIH Center with human prostate tissue explants in the RWV Bioreactor to begin to investigate basic prostatic epithelial pathobiology. It was found that rat prostate tissue explants could be maintained in the RWV bioreactor, and the epithelial cells in the tissue blocks showed a high proliferative activity. In one experiment, the addition of phenylephrine to the culture media led to the formation

of histological abnormalities within the RWV cultured tissue which resembled the *in vivo* data derived from rats injected with phenylephrine. The RWV Bioreactor maintained normal human prostate tissue without gross cytoarchitectural change. The RWV bioreactor has demonstrated mixed results in its ability to culture prostatic carcinoma cells from biopsy samples.

Evaluating the Role of Gap Junctions in Lymphoid Tissue in RWV Bioreactor. A. Harris, Johns Hopkins University & NASA/NIH Center, has evaluated the role of gap junctions in the function of human lymphoid tissue cultured in RWV. Connexin 43-positive cells were identified in human tonsillar tissue. Various chemically unrelated inhibitors of gap junctions affect the ability of tissue to respond to recall antigens by production of specific antibodies.

Several other groups (LEP, NIEHS, LCMB, NICHD; DCT, NCI, and USAMRIID) have continued their projects with the RWV to study HIV infection in human tonsillar tissue, to monitor the development of cartilage, to investigate the role of normal fibroblasts in development of uterine leiomyomas, to obtain pure cultures of human mononuclear phagocytes for cancer therapy, to assess the effect of microgravity on lymphoid tissue immune function, as well as to develop the RWV into a "universal" pathogen culture system.

LABORATORY OF CELLULAR AND MOLECULAR BIOPHYSICS

Chief
Joshua Zimmerberg, M.D., Ph.D.

Principal Investigators
Andreas C. Chrambach, Ph.D.
Alfred L. Yergey, Ph.D.
Leonid Chernomordik, Ph.D.

Tenure-Track Investigator
Leonid Margolis, Ph.D.

Senior Fellows
Paul Blank, Ph.D.
Alexandr Chanturiya, Ph.D.
Svetlana Glushakova, Ph.D.
Andrew Harris, Ph.D.
Jeffrey Kowalak, Ph.D.

Post-Doctoral Fellows
Austin Bailey, Ph.D.
Gorka Basanez, Ph.D.
Xingguo Chen, Ph.D.
Nong Chen, Ph.D.
Jens Coorssen, Ph.D.
Jing Fan, M.D.
Jean-Charles Grivel, Ph.D.
Steven Hatfill, M.D., Ph.D.
David Kingsley, Ph.D.
Irina Kolosova, Ph.D.
Ingrid Markovic, Ph.D.
Viet Quoc Nguyen, Ph.D.
Sergey Radko, Ph.D.
Andrew Sylwester, Ph.D.
Masahiro Tahara, M.D., Ph.D.

Guest Researchers
Peter Backlund, Ph.D.
Boris Baibakov, Ph.D.
Valerij Chestkov , Ph.D.
Wendy Fitzgerald, Ph.D.
Steven Hollenberg, M.D.
Mukesh Kumar, Ph.D.
Kimberly O'Brien, Ph.D.
Ilya Plonsky, Ph.D.
Helena Pulyaeva, Ph.D.
Vladimir Ratinov, Ph.D.
Kim Timmers, Ph.D.
Timothy Whalley, Ph.D.
Elena Yarmola, M.D., Ph.D.
Shu-Rong Yin, M.D.

Pre-Doctoral Fellows
Afshin Rashtian
Joshua Ward

Senior Technical Specialist
Nancy Vieira

ANNUAL BIBLIOGRAPHY

Acevedo LD, Xu Y, Zhang X, Pierce RJ, Yergey AL. Quantification of acetylcholine in cell culture systems by semi-micro high-performance liquid chromatography and electrospray ionization mass spectrometry. J Mass Spectrom 1996;31:1399-402.

Chang H-T, Chen N, Sullivan J V, Chrambach A. Preparative electrophoresis in a concentrated polymer solution: automated procedure for microsome isolation. Anal Biochem 1996; 47:111-4.

Chanturiya AN. Filter switching device for dual wavelength video imaging. J Fluroresc 1996;6:103-6.

Chanturiya AN, Nikolaenko AN, Shatursky OYo, Lishko VK. Probing the structure-function relationship of α-latrotoxin formed channels with antibodies and pronase. Toxicon 1996;34:1157-64.

Chen N, Chrambach A. Isolation of subcellular-sized particles separated by electrophoresis in dilute polymer solution, using commercial electrophoresis apparatus with intermittent scanning of fluorescence. Electrophoresis 1997;18:781-3.

Chen N, Chrambach A. Improved resolution in the gel electrophoresis of proteins by a periodically interrupted electric field. J Biochem Biophys Methods 1996;33:163-70.

Chen N, Chrambach A. Application of the commercial gel electrophoresis apparatus with intermittent fluorescence scanning to a non-fluorescing protein. Anal Biochem 1996;242:64-7.

Chen N, Chrambach A. The resolution between two native proteins and between their SDS-complexes in agarose and polyacrylamide gel electrophoresis. Electrophoresis 1997;18:1126-32.

Chen XG, Brining SK, Nguyen VQ, Yergey AL. Simultaneous assessment of conformation and aggregation of β-amyloid peptide using electrospray ionization mass spectrometry. FASEB J 1997;11:817-23.

Chernomordik LV. Non-bilayer lipids and biological fusion intermediates. Chem Phys Lipids 1996;81:203-13.

Chernomordik LV, Leikina E, Frolov V, Bronk P, Zimmerberg J. An early stage of membrane fusion mediated by low pH conformation of influenza hemagglutinin depends upon membrane lipids. J Cell Biol 1997;136:81-94.

Fitzgerald RL, Herold DA, Yergey AL. Trade-offs in mass spectrometry [editorial; comment]. Clin Chem 1997;43:915.

Freeman SPHT, King JC, Vieira NE, Woodhouse LR, Yergey AL. Human calcium metabolism including bone resorption measured with 41Ca tracer. Nucl Instrum Meth B 1997;123:266-70.

Galkina SI, Sudina GF, Margolis LB. Regulation of intracellular pH by phospholipase A 2 and protein kinase C upon neutrophil adhesion to solid substrata. FEBS Lett 1996;393:117-20.

Glushakova S, Baibakov B, Zimmerberg J, Margolis LB. Experimental HIV infection of human lymphoid tissue: correlation of CD4+ T cell depletion in and virus syncytium-inducing/non-syncytium inducing phenotype in histocultures inoculated with laboratory strains and patient isolates of HIV type 1. AIDS Res Hum Retroviruses 1997;13:461-71.

Goans RE, Weiss GH, Vieira NE, Sidbury JB, Abrams SA, Yergey AL. Calcium kinetics in glycogen storage disease type 1a. Calcif Tissue Int 1996;59:449-53.

Margolis L, Fitzgerald W, Glushakova S, Hatfill S, Amichai S, Baibakov B, Zimmerberg J. Lymphocyte trafficking and HIV infection of human lymphoid tissue in rotating wall vessel bioreactor. AIDS Res Retroviruses 1997;13:1411-20.

Mauras N, Vieira NE, Yergey AL. Estrogen therapy enhances calcium absorption and retention and diminishes bone turnover in young girls with Turner's syndrome: a calcium kinetic study. Metabolism 1997;46:908-13

Melikyan GB, Chernomordik LV. Membrane rearrangements in fusion mediated by viral proteins. Trends in Microbiol 1997;5:1-10.

Nguyen VQ, Chen XG, Yergey AL. Observations of magic numbers in gas phase hydrates of protonated amines and tetraalkyl ammonium ions. J Am Soc Mass Spect 1997;8:1175-9.

Plonsky I, Zimmerberg J. The initial fusion pore induced by baculovirus GP64 is large and forms quickly. J Cell Biol 1996;135:1831-9.

Radko SP, Chrambach A. Mechanisms of retardation of rigid spherical particles with 3 to 1,085 nm radius in capillary electrophoresis, using buffered polyacrylamide (Mr = 5 million) solutions. Electrophoresis 1996;17:1094-102.

Radko SP, Chrambach A. Electrophoresis of proteins in semidilute polyethylene glycol solutions: mechanism of retardation. Biopolymers 1997;42:183-9.

Radko SP, Chrambach A. Electrophoresis in polymer solutions: mechanisms of "molecular sieving". J Phys Chem 1996;100:19461-5.

Radko S P, Weiss G H, Chrambach, A. Protein band spreading in CZE: effects of sample zone length and presence of polymer. J Chromatography A 1997;781:277-286.

Weiss GH, Sokoloff H, Zakharov SF, Chrambach A. Interpretation of electrophoretic band shapes by a partition chromatographic mo del. Electrophoresis 1996;17:1325-32.

Yarmola E, Calabrese PP, Chrambach A, Weiss, GH. Interaction with the matrix: the dominant factor in macromolecular band spreading in gel electrophoresis. J Phys Chem 1996;101:2381-7.

Yarmola E, Chrambach A. Improved sensitivity of detection by the HPGE-1000 apparatus through modification of its optical system. Electrophoresis 1997;18:1951-4.

Yarmola E, Sokoloff H, Chrambach A. The relative contributions of dispersion and diffusion to band spreading (resolution) in gel electrophoresis as a function of DNA length. Electrophoresis 1996;17:1416-9.

LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY

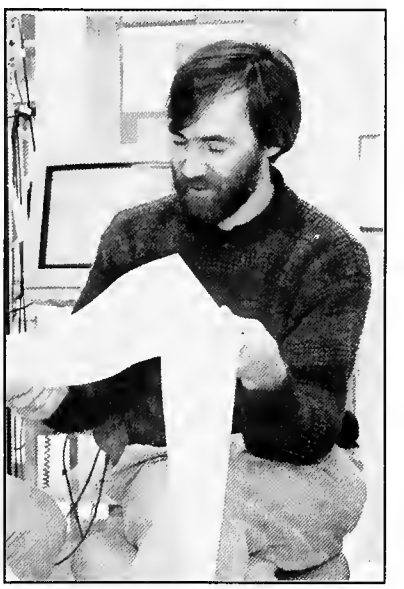
Mark L. Mayer, Ph.D., Chief

The Laboratory of Cellular and Molecular Neurophysiology (LCMN) studies receptors and ion channels in the mammalian central nervous system. Investigators in this laboratory use the techniques of neurophysiology and biophysics, molecular biology, and calcium imaging to analyze a wide variety of brain processes, such as the properties of individual receptors and channels, mechanisms underlying synaptic plasticity, and the regulation of gene expression in neurons and glia. Projects that combine the skills of individual investigators, and the overlapping interests of staff in the laboratory, facilitate a collaborative atmosphere.

STRUCTURE AND FUNCTION OF GLUTAMATE RECEPTORS

Excitatory synaptic transmission throughout the brain is mediated by activation of a complex family of glutamate receptors, which are assembled from subunits encoded by the AMPA, kainate, and NMDA receptor gene families. The Section of Neurophysiology and Biophysics, under the direction of ***Mark Mayer***, investigates the properties of glutamate receptor ion channels, using electrophysiological approaches to study ion channel structure and function. In the past year, work focused on two main topics: allosteric modulation of AMPA receptors; and the channel blocking action of polyamines.

A Novel Allosteric Regulator of AMPA Receptors. AMPA receptors that mediate fast synaptic transmission throughout the CNS exist in two major splice variants (flip and flop), each with different functional properties. The identification of cyclothiazide as a flip-preferring modulator of desensitization and deactivation provided a useful tool for the study of AMPA receptor function but highlighted the need for a modulator with selectivity for flop splice variants. In collaboration with Masayuki Sekiguchi of the National Institute of Neuroscience, Tokyo, we have now analyzed the action of a new allosteric modulator, PEPA (4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide), which meets this requirement.



Mark Mayer

Experiments performed using *Xenopus* oocytes for dose-response analysis revealed 50-fold potentiation by 200 μM PEPA for the flop variant of the glutamate receptor subunit GluRCflop but only three-fold potentiation for GluRCflip. To determine whether the selectivity of PEPA for flop versus flip variants also holds for other AMPA receptor subunits, we compared potentiation of responses to glutamate by 200 μM PEPA for GluRA, GluRC, and GluRD, expressed alone and in combination with GluRB. PEPA consistently causes greater enhancement of currents evoked by 100 μM glutamate in oocytes expressing AMPA receptor flop versus isoforms compared with flip isoforms for all subunits examined. However, there are clear differences between subunits, and between homomeric receptors and heteromeric receptors generated by coassembly with GluRB. GluRAflop is the least sensitive of the flop variants. Coassembly with GluRBflop increases potentiation by PEPA for GluRAflop and for GluRCflop. In heteromeric receptors formed from GluRB and GluRC, the magnitude of enhancement by PEPA depends on the splice isoform of GluRC; while we observed marked potentiation for GluRBflopCflop, GluRBflipCflop is only moderately potentiated, and GluRBflipCflip and GluRBflopCflip are only weakly potentiated.

When compared with 1 mM aniracetam and 25 $\mu\mu$M cyclothiazide, two well characterized allosteric modulators of AMPA receptors, 25 μM PEPA causes much greater potentiation of glutamate responses in oocytes expressing GluRCflop. In contrast, cyclothiazide causes the greatest potentiation in oocytes expressing GluRCflip. In an attempt to accurately define differences in the potency for modulation of AMPA receptors, we performed dose-response analyses. The EC_{50} for potentiation of GluRCflop by PEPA is 50 μM (Hill coefficient = 1.03). Aniracetam does not produce any detectable potentiation at 100 μM and, at 2 mM, potentiation is comparable to that produced by 10

μM PEPA. Complete dose-response curves could not be constructed for aniracetam due to its limited solubility and low potency, but our data indicate that PEPA is at least 200-times more potent for potentiation of GluRCflop than aniracetam.

PEPA Suppresses AMPA Receptor Desensitization. A widely recognized limitation of the use of two-electrode voltage clamp recording from oocytes is that it is impossible to apply solutions rapidly enough to resolve the very rapid desensitization exhibited by AMPA receptors. To address this issue, we employed whole-cell recording with rapid perfusion to study the effects of PEPA on recombinant AMPA receptor responses in transiently transfected 293 cells. Such analysis revealed that differences in the extent of desensitization of control responses contribute to the extent of potentiation by PEPA. For example, although PEPA nearly completely blocks desensitization for both GluRBflopDflop and GluRBflipDflip, potentiation of equilibrium responses to glutamate for these subunits suggests strong flop selectivity (GluRBflopDflop 75-fold potentiation; GluRBflipDflip 10-fold potentiation). Subunit combinations, such as GluRBflopDflop, that show the greatest extent of desensitization at equilibrium are much more strongly potentiated by PEPA than are subunit combinations such as GluRBflipDflip, which show less desensitization of control responses to glutamate.

Kinetic Analysis of AMPA Receptor Modulation. The rate of onset of desensitization in the presence of PEPA demonstrates both selective modulation of flop splice variants, as well as subunit-dependent effects of PEPA. Thus, even for GluRA, the least sensitive subunit examined, for which desensitization remains pronounced in the presence of PEPA for both flip and flop splice variants, the time constant of onset of desensitization for GluRAflop (τ 250 ms) is slowed by 100 μM PEPA nearly three times more than for GluRAflip (τ 90 ms). The flop-selective action of PEPA is further underscored by the complete block of desensitization by 100 μM PEPA for both GluRDflop and GluRAflopBflop, but not for GluRDflip and GluRAflipBflip. However, we found no clear difference in the extent of attenuation of desensitization by 100 μM PEPA between GluRCflop versus GluRCflip and GluRBflopDflop versus GluRBflipDflip, even though the subunit dependence for slowing of desensitization follows the rank order GluRD > GluRC > > GluRA.

A feature of allosteric modulation by PEPA, which is common also to aniracetam and cyclothiazide, is that each drug potentiates AMPA receptor currents by suppressing desensitization. However, on the basis of prior work, we proposed that aniracetam and cyclothiazide suppress desensitization by different mechanisms. Kinetic modeling of the rapid deactivation and desensitization kinetics of GluRAi and GluRAo suggest that aniracetam slows the rate of channel closing, indirectly slowing the onset of desensitization, whereas cyclothiazide has a direct effect on the rate constant of desensitization, and in addition stabilizes agonist-bound closed states, thus increasing agonist affinity and slowing deactivation. Additional experiments are now required to develop a kinetic model for the action of PEPA.

Polyamines Permeate Glutamate Receptor Ion Channels. Ion channel block by cytoplasmic polyamines for inward rectifier potassium channels differs from that for glutamate receptors. For potassium channels, there is no relief from block at positive potentials, while glutamate receptors show biphasic IVs (current/voltage plots), indicating that polyamines are likely to be weakly permeable. To explore this possibility, we used outside out patches for GluR6(Q) channels with bi-ionic recording conditions where polyamines were sole-charge carriers on the external side of the membrane. We detected small inward membrane currents reliably with putrescine, spermidine, and in some patches spermine, confirming that polyamines can permeate GluR6(Q) channels. Reversal potential estimates for polyamine currents give permeability ratios relative to Na^+ of 0.42 and 0.07 for putrescine and spermidine respectively; we calculated these using the sum of the GHK (Goldman/ Hodgkin/Katz) constant field current equations for individual ionic species to interpolate the permeability ratio required to give measured reversal potentials.

Consistent with the idea that relief of block by cytoplasmic polyamines observed at positive potentials is due to permeation, experiments performed last year showed that spermine and two N-substituted analogs, 4-OH-phenyl-propanoyl spermine and Philanthotoxin 343 (PhTX), display different degrees of relief from block, which was attributed to differences in their molecular dimensions. Relief from block observed with spermine, which has a cross-sectional diameter of 0.16 nm^2, is the most pronounced, while for PhTX, which has a cross-sectional diameter of 0.44 nm^2, we were unable to detect any permeation. Our demonstration that endogenous polyamines can permeate GluR6(Q) channels and that this accounts for relief of block is consistent with recent pore size estimates by Sakmann's laboratory (MPI Heidelberg) of a channel diameter of 0.44 nm^2.

Kinetic Analysis of Block by Polyamines. To address the issue of whether polyamine block could occur sufficiently rapidly to influence responses at central synapses, we attempted to measure the rate constants for open channel

block using voltage-jump experiments. In the presence of internal spermine, spermidine, and PhTX, with domoic acid and concanavalin A used to achieve a high open probability at equilibrium, the current elicited by depolarizing voltage steps from -100 mV decays exponentially to a new steady-state level. Current relaxations were not observed in the absence of internal polyamines, suggesting that the current decay reflects the time course of polyamine block. The rate of onset of block increases with blocker concentration and, at +35mV, plots of the reciprocal of the time constant for block against blocker concentration are linear, consistent with a bimolecular reaction scheme, in which the blocker enters and occludes open channels. The rate constant for binding of spermine, spermidine, and PhTX, estimated from the slope versus blocker concentration, is 9.0×10^7, 5.3×10^7, and 1.9×10^7 M^{-1} s^{-1} respectively. At physiological pH, spermine and spermidine have net valencies close to +4 and +3 respectively, which may account for their different entry rates into the channel. The even slower rate for PhTX, which is a bulky analog of spermidine, may reflect the requirement for binding in a particular orientation for block to occur. The voltage-dependence for the rates of binding (e-fold per 168 mV) and unbinding (e-fold per -25 mV) for 100μM PhTX suggests that the voltage dependence of block is almost entirely due to the unbinding rate. This could be explained by an asymmetrical inner barrier, such as occurs for Mg^{2+}-block of ATP-dependent K^+-channels. Thus, our results to date are consistent with a rapid onset of block at physiological concentrations of polyamines, which could well be established during synaptic transmission. Further analysis of these experiments is proceeding with a simple 2 barrier-1 site model to estimate the rate constants of permeation for individual polyamines.

Structure-Function Analysis of Polyamine Block. The pore region of polyamine-sensitive glutamate receptors is subject to a novel form of regulation termed RNA editing, in which an adenosine deaminase alters a single codon from CAG to CIG, substituting Arg for Gln (R/Q site). In edited homomeric receptors, the positively charged ring of arginine residues in the pore-forming region abolishes block by polyamines, either by repulsion of positively charged polyamine molecules approaching this site, or by disrupting a binding site for polyamines, for example by forming a salt bridge with a nearby negatively charged residue, which normally binds to polyamines, as proposed by Dingledine and colleagues. However, the extended structure of polyamines, in which the terminal amine groups of spermine are separated by 1.7 nm, makes it unlikely that binding within the channel occurs at a single site. To address this issue, we are constructing and analyzing a series of mutants with the aim of identifying sites involved in polyamine regulation.

In GluR6, RNA editing occurs at position 590 in a region termed M2, which is thought to form the narrowest region of the pore; the vestibule of the pore is likely to be formed in part by residues in the membrane-spanning sequences M1, M3, and perhaps M4. To address the role of the residue at position 590, we inserted Gly, Ala, Leu, Ser, Cys, Thr, Met, Asn, Asp, Glu, His, Lys, and Arg, and compared responses to kainate with those for GluR6(Q), using *Xenopus* oocytes as an expression system. Following treatment with concanavalin-A to block desensitization, we performed Boltzman analysis to measure the voltage dependence for onset of block, and in addition determined the extent of maximum block at positive membrane potentials, as well as relief from block at +80 mV, and the conductance at -80 mV as an indicator of changes in expression or impaired permeability to Na^+. The results of such substitutions show, surprisingly, that at the Q/R site introduction of Asp and, to a lesser extent Glu, strongly reduces rectification resulting from polyamine block. Thus, maximum block is reduced from 98% for WT to 48% for Q590D; relief from block, measured as the conductance ratio at +80mV/-80mV, increases from 0.10 (WT) to 1.2 (Q590D) and 0.4 (Q590E). This result is unexpected if, as has been convincingly demonstrated for NMDA receptors, the Q/R site indeed forms the narrowest region of the pore, since a negatively charged residue at this site would be expected to interact strongly with polyamines entering the pore. In fact, rectification for Q590D is similar to that for Q590S, i.e., maximum block 43%, conductance ratio at +80mV/-80mV being 1.21, suggesting that size rather than charge *per se* markedly influences permeation and block by polyamines. This is supported by two additional observations: in Q590N, the smaller Asn residue gives lower maximum block (40%) and greater relief from block (ratio +80mV/-80mV = 0.68) than the Gln present in wild-type receptors, while for the hydrophobic series Gly, Ala, Leu, maximum block increases and relief from block decreases with size of the side chain. In the case of the positively charged side chain series His, Lys, Arg, we found weak inward rectification for His, and weak outward rectification for Lys and Arg, suggesting that the lower pKa of the His side chain does not generate sufficient positive charge to fully block binding to polyamines.

Ongoing work includes an alanine scan of flanking position 590 and analysis of mutants based on a model of the pore forming region of the GluRA subunit that includes regions likely to form part of the external vestibule of the pore.

HIPPOCAMPAL INTERNEURONS AND THEIR ROLE IN CONTROLLING EXCITABILITY

Although GABA-containing inhibitory neurons represent only 10% of the total hippocampal neuron population, their inhibitory synaptic tone onto pyramidal neurons far outweighs synaptic excitation. Despite the wealth of information available about inhibitory synaptic events received by pyramidal neurons, little is known about interneurons, beyond the basic physiology of the varied cell types from which inhibition arises. A major effort in the Unit on Cellular and Synaptic Neurophysiology, headed by ***Chris McBain***, is to understand the ionic mechanisms that regulate the activity of inhibitory neurons and to learn how these mechanisms impact hippocampal function under both physiological and pathological conditions. In addition, we are studying the participation of inhibitory neurons in the plastic phenomenon of long-term potentiation (LTP) or depression and the effects of changes in the synaptic input to these cells on the hippocampal network.

A Lack of Presynaptic LTP at Mossy Fiber Synapses onto Interneurons. Mossy fiber terminals arising from dentate gyrus granule cells innervate both inhibitory neurons and pyramidal neurons of the CA3 hippocampus. Synaptic plasticity at the mossy fiber terminals onto pyramidal neurons of the CA3 subfield is entirely pre-synaptic in origin and requires an elevation of intracellular calcium and cAMP formation in the presynaptic mossy fiber terminals. We have designed experiments to determine whether the same mossy fibers that contact CA3 pyramidal neurons and *stratum lucidum* cells possess identical presynaptic forms of LTP. We found that tetanic stimulation of mossy fibers induces a presynaptic form of LTP in pyramidal neurons, but is without effect, or induces depression at synapses onto interneurons. Unlike, pyramidal neurons, synaptic transmission onto interneurons is not potentiated following cAMP formation. Furthermore, metabotropic glutamate receptor (mGluR) modulation of neurotransmission onto interneurons occurs preferentially through a protein kinase C-mediated pathway. At pyramidal neuron synapses, mGluR modulation of synaptic transmission occurs through both cAMP- and PKC-dependent pathways. Thus, the functional properties of mossy fiber terminals vary, depending on their postsynaptic target neuron. The differential cAMP sensitivity would ensure that synaptic efficacy is either unaltered or depressed at presynaptic terminals onto interneurons during protocols that induce LTP at principal cell synapses. These data extend our previous studies and strengthen the hypothesis that interneurons lack the machinery necessary for synaptic plasticity induction.

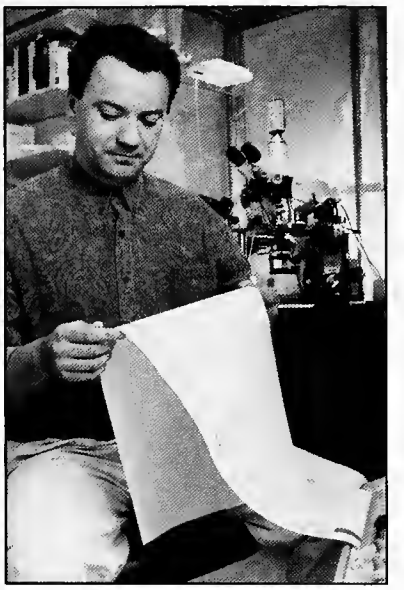
Ca^{2+} Permeability of AMPA Receptor EPSCs in Single CA3 Interneurons. Both Ca^{2+} -permeable and Ca^{2+} -impermeable subtypes of AMPA receptors have been previously demonstrated on CA3 interneurons. The origins of the afferent inputs to these receptors, however, are completely unknown. In addition, no information exists as to whether both receptor subtypes are present on a single cell. We have used PhTX, a toxin isolated from the Digger Wasp, which selectively blocks Ca^{2+}-permeable AMPA receptors, to determine the nature of AMPA receptors of *stratum lucidum* interneurons innervated by either dentate granule cell mossy fibers or CA3 recurrent collaterals. Mossy fibers make synapses onto both calcium-permeable/PhTX-sensitive and calcium impermeable AMPA receptors. In contrast, synaptic inputs from CA3 pyramidal cell fibers are exclusively through synapses comprising calcium-impermeable receptors. In addition, single *stratum lucidum* interneurons receive inputs from both mossy fibers and commissural/associational axons onto calcium-ion-permeable and calcium-ion-impermeable AMPA receptors, respectively. These data underscore the level of complexity of integration of afferent inputs onto single hippocampal neurons and complicate interpretation of experiments designed to correlate function with mRNA expression.

The Subcellular Distribution of the K Channel Subunit Kv2.1 in Interneurons. A variety of voltage-gated ion channels are expressed on principal cell dendrites, and it has been suggested that they play a role in the regulation of dendritic excitability. We have shown, using double immunostaining, that Kv2.1 protein is expressed in the majority of cortical and hippocampal parvalbumin-, calbindin-, and somatostatin-containing inhibitory interneurons. At the electron microscopic level, Kv2.1 immunoreactivity was primarily observed on the plasma membrane of the somata and proximal dendrites of both principal neurons and inhibitory interneurons; expression is low on smaller dendritic branches, and absent from axons and presynaptic terminals. The Kv2.1 subunit is highly expressed on the cell surface membrane immediately facing astrocytic processes. Kv2.1 expression is also concentrated in specific cytoplasmic compartments and on the subsurface cisterns underlying the plasma membrane facing astrocytes. In addition, Kv2.1 subunit immunoreactivity is associated with postsynaptic densities of a fraction of inhibitory symmetric synapses, while expression at asymmetric synapses is rare.

These data demonstrate that channels formed by Kv2.1 subunits are uniquely positioned on the soma and principal dendrites of both pyramidal cells and inhibitory interneurons at sites immediately adjacent to astrocytic processes.

This close apposition to astrocytes would ensure a rapid removal and limit the influence of K^+ released into the extracellular space. This expression pattern suggests that channels formed by Kv2.1 are positioned to play a role in the regulation of neuronal dendritic excitability.

The potassium channel subunit Kv2.1 is widely expressed in most CNS neurons, suggesting that channels formed by this subunit may give rise to a "generic" delayed rectifier current. Kv2.1 channel function has been primarily studied in transfected or mRNA-microinjected systems, while no studies of native channels containing Kv2.1 have been performed. We have devised experiments aimed at determining the role of Kv2.1-containing channels in pyramidal neurons using antisense oligonucleotides to selectively knock out Kv2.1 subunit expression. Western blot analysis revealed that total Kv2.1 protein content decreases ~90% after antisense treatment. Consistent with a reduction in the Kv2.1 protein content, delayed rectifier currents in pyramidal neurons are markedly reduced. In neurons bathed in physiological concentrations of $[K^+]_o$, no discernible differences were observed in action potential waveforms evoked by somatic depolarization in either antisense-treated, control untreated, or non-treated cells. However, elevation of $[K^+]_o$, a condition that precipitates electrographic seizures, results in a prolonged broadening of action potentials in antisense-treated neurons. This suggests that, under physiological concentrations of $[K^+]_o$, the repertoire of currents that remain in antisense-treated cells is sufficient to permit normal action potential repolarization. During electrographic seizure events, however, currents through Kv2.1 appear to be essential for spike repolarization.


Chris McBain

In antisense-treated cells, action potential waveforms activated from suprathreshold EPSPs are highly-frequency dependent. At low stimulation frequencies (0.2 Hz), trains of action potentials evoked by suprathreshold EPSPs are identical. At higher frequencies (1 Hz), however, the repolarizing phase of each subsequent action potential broadens until inadequate repolarization occurs and a plateau depolarization is observed. These data suggest that potassium channels expressed on the cell dendrites have an important role to play in the fidelity of high frequency synaptic transmission.

Arachidonic Acid Modulates Transient Outward Potassium Currents. We studied the transient outward potassium current in outside-out macropatches excised from the soma of CA1 pyramidal neurons and *stratum oriens-alveus* inhibitory interneurons in rat hippocampal slices. We found that arachidonic acid (AA) dose-dependently decreases the charge transfer associated with the transient current, concomitant with an increase in the rate of current inactivation. AA does not affect the voltage-dependence of steady-state inactivation but does prolong the time required for complete recovery from inactivation. The effects of AA are mimicked by the non-metabolizable analog of AA, ETYA, suggesting that metabolic products of AA are not responsible for the observed blocking action. In addition, AA blocks stratum OA-LM interneuron transient currents but not currents recorded from basket cell interneurons. In current clamp experiments, AA is without effect on the action potential waveform of CA1 pyramidal neurons under control recording conditions. In voltage-clamp experiments, the use of a test pulse paradigm, designed to mimic the action-potential voltage-trajectory, reveals that the transient current normally associated with a single spike deactivates too rapidly for AA to have an effect. Transient currents activated by longer duration "action potential" waveforms are, however, attenuated by AA. Consistent with this finding was the observation that AA broadens interictal spikes recorded in the elevated $[K^+]_o$ model of epilepsy. These data suggest that AA liberated from hippocampal neurons may selectively block the transient current in both CA1 pyramidal neurons and inhibitory interneurons and selectively broaden action potentials under pathological conditions.

Oligodendrocyte Proliferation and Potassium Channel Function. In a continuing collaboration with Vittorio Gallo's laboratory (LCMN), we have investigated the role of acute and chronic β-adrenergic receptor activation and cAMP formation on proliferation mechanisms in cells of the oligodendrocyte lineage. We found that acute activation of β-adrenergic receptors does not alter K^+ channel function in O-2A cells. Chronic exposure to β-adrenergic agonists prevents O-2A cell proliferation by a mechanism involving long-term modulation of the voltage-dependence of delayed rectifier K currents, possibly by a mechanism involving cAMP formation. Chronic treatment with agents that elevate cAMP levels mimics the effects of chronic exposure to β-adrenergic agonists. This simple shift of the voltage-dependence of current activation would effectively reduce the amount of available

outward current during depolarizations around the resting membrane potential, limiting the magnitude of the repolarization, thus permitting a greater depolarization for a given stimulus. This mechanism is essentially equivalent to the frank reduction of K channels caused by other antiproliferative agents.

Social Interaction and Sensorimotor Gating Abnormalities in Mice Lacking *Dvl1*. The *wingless/Wnt* pathway is a highly conserved developmental pathway involved in cell fate determination in eukaryotic organisms. Disruption of *Wnt1* results in midbrain-hindbrain defects. *Dishevelled (dsh)*, a segment polarity gene first cloned in *Drosophila*, is absolutely required cell-autonomously for *wg* signaling. In the mouse, three closely related *dsh* genes have been isolated. The region of highest conservation contains the PDZ motif, present in many proteins localized to tight junctions and cytoskeletal structures. No known biochemical function, however, has been identified for any of the *dsh* proteins. The *dsh* PDZ domain region is required for *wg*-mediated hyperphosphorylation. In a collaboration with Wynshaw-Boris of the Human Genome Research Institute, we have studied long-term potentiation in mice completely deficient for Dvl1, created by gene targeting. Dvl1 mice are viable, fertile, and structurally normal. Surprisingly, these mice exhibit reduced social interaction, including differences in whisker trimming, deficits in nest building, less huddling contact, and subordinate responses in social dominance tests. Sensorimotor gating is abnormal, as measured by deficits in acoustic and tactile startle. In electrophysiological experiments using *in vitro* hippocampal slices, field excitatory postsynaptic potentials were elicited by stimulating *stratum radiatum* afferents. Using conventional LTP induction protocols, the percentage of potentiation in wild-type and *Dvl1* mice is comparable. Likewise, paired pulse facilitation is identical in both genotypes, suggesting that Dvl1-deficient mice have normal synaptic plasticity. Overall, the results from the Dvl1 mutant provide a model for aspects of several human psychiatric disorders. These results are consistent with an interpretation that common genetic mechanisms underlie abnormal social behavior and sensorimotor gating deficits, and implicate Dvl1 in processes underlying complex behaviors.

ION CHANNELS AND RECEPTORS IN GLIAL CELLS

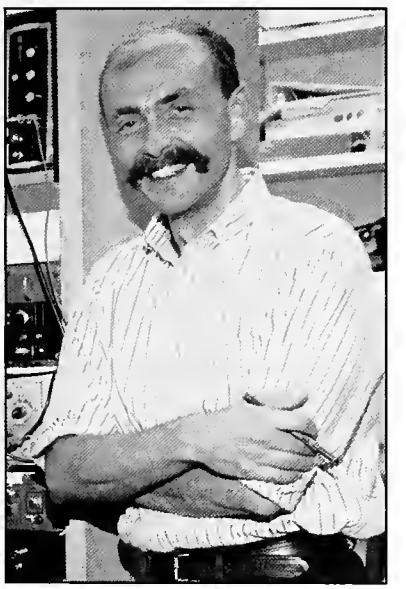
The Section on Molecular Neurobiology of Glia, led by ***Vittorio Gallo***, studies the molecular and functional properties of neurotransmitter ligand-gated ion channels and receptors in glial cells. Our current emphasis is on the physiological role of glutamate and adrenergic receptors during glial development, and on the regulation of glutamate receptor gene expression in the brain.

Physiological Role of Glutamate Receptors in Oligodendrocyte Development. In culture, oligodendrocyte progenitor (O-2A) cells differentiate to oligodendrocytes with a time schedule similar to that *in vivo*. We hypothesized that the neurotransmitter glutamate may regulate oligodendrocyte development through the activation of membrane receptors expressed early during O-2A proliferation and migration. Experiments in cultured cerebellar cells and in tissue slices were performed to verify this hypothesis. Platelet-derived and basic fibroblast growth factors (PDGF and bFGF, respectively) regulate oligodendrocyte progenitor (O-2A) proliferation, migration, and differentiation *in vitro* and *in vivo*. We found that proliferation of cerebellar O-2 progenitors maintained in culture with PDGF or bFGF is inhibited by AMPA-preferring glutamate receptor (GluR) agonists. Electrophysiological experiments performed in collaboration with Chris McBain and his colleagues demonstrated that activation of GluRs in cerebellar O-2A cells causes a reversible block of voltage-dependent delayed rectifier K^+ channels. Direct blockage of delayed rectifier K^+ channel with tetraethylammonium (TEA), treatment with the Na^+ channel opener veratridine, or incubation with high (45 mM) extracellular concentrations of K^+ ions also inhibit O-2A progenitor proliferation. Activation of GluRs prevents cerebellar O-2A lineage progression to the pro-oligodendroblast stage, as monitored with the monoclonal antibody O4. These findings indicate that cerebellar oligodendrocyte progenitor proliferation and development is regulated by GluR agonists through a Na^+-dependent blockage of delayed rectifier K^+ channels.

The role of GluR in oligodendrocyte development was also analyzed in a more intact system. Postnatal day 2-10 (P2-P10) cerebellar slices were labeled with bromodeoxyuridine (BrdU) to monitor proliferation of different cell populations. After acute dissociation, cells were stained with antibodies for different oligodendrocyte antigens that are developmentally-regulated. In agreement with previous *in vivo* studies, the number of $O4^+$ pro-oligodendroblasts and $O1^+$ oligodendrocytes significantly increases in cerebellar slices between P6 and P10. The maximal increase in $O1^+$ cells (at P10) is preceded by a peak of replication of $O4^+$ cells at P6. The total number of O-2A progenitors/cerebellum (stained with the LB1, NG2 and A2B5 antibodies) does not significantly change between P2 and P10, but their proliferative potential displays a four-fold decrease during the same time period.

Cerebellar slices were treated with GluR agonists or antagonists. Kainate and AMPA significantly reduce the percentage of $LB1^+$, $NG2^+$, $A2B5^+$ and $O4^+$ cells, and the BrdU labeling index in all these cell types. Numbers of $O1^+$ cells are also reduced. The non-NMDA GluR antagonist DNQX has opposite effects on all cell populations. The percentage of astrocytes is not altered by GluR agonists or by DNQX. Consistent with the findings obtained with antigenic markers, mRNA transcript levels of the oligodendrocyte-specific gene CNP declines in P6 slices after treatment with kainate and AMPA, and rises with DNQX treatment. Treatment with the NMDA receptor antagonist APV does not modify the percentage of $O4^+$ cells, nor their BrdU incorporation. Finally, incubation with the GABA receptor antagonist bicuculline does not affect oligodendrocyte development. We conclude that glutamate selectively inhibits O-2A progenitor proliferation and lineage progression in cerebellar slices through activation of AMPA receptors and most likely through a blockage of voltage-dependent K^+ channels.

Beta-Adrenergic Receptors in Oligodendrocyte Lineage Cells and Their Role in Development. O-2A cells purified from the rat cerebral cortex express adrenergic receptors of the α and β subtypes. Calcium imaging experiments performed in collaboration with James Russell's group demonstrated that only norepinephrine (NE) and the α1-selective agonist phenylephrine produce significant intracellular Ca^{2+} transients in these cells. Activation of β-adrenergic receptors with the selective agonist isoproterenol (Iso) or with NE reversibly inhibits O-2A cell proliferation stimulated by PDGF and/or bFGF. Alpha-adrenergic receptor agonists are ineffective. Treatment of O-2A cells with Iso raised by 40-50% the proportion of $O4^+$ cells in the cultures. Iso and NE strongly augment (30-fold over baseline) intracellular cAMP levels in O-2A cells, whereas α-adrenergic agonists are ineffective. The effects of Iso can be mimicked by 8-Br-cAMP, which inhibits O-2A cell proliferation and enhances differentiation. In synchronized O-2A cells, Iso causes an arrest in G1 phase of the cell cycle. Similarly to GluR agonists, Iso drastically reduces the percentage of cells that enter S-phase, but is ineffective when applied during S-, G_0, or M-phase.


Vittorio Gallo

In view of our previous work that indicated an involvement of O-2A K^+ channels in cell proliferation, the effects of noradrenergic agonists and 8-Br-cAMP on K^+ currents were analyzed in collaborative experiments with McBain's group. Acute application of Iso, 8-Br-cAMP or α-adrenergic agonists does not affect sustained or transient K^+ currents. However, in cells cultured with Iso or 8-Br-cAMP, a rightward shift in the voltage-dependence of activation was observed. Alpha-adrenergic agonists have no effects. Our experiments demonstrate that β-adrenergic receptor activation modulates O-2A cell proliferation through a mechanism involving K^+ channel plasticity. Combined with our results obtained with GluR agonists, these experiments also indicate that arrest in O-2A cell proliferation can result either in retarded or accelerated differentiation. Multiple neurotransmitter signals can therefore act in concert with other cellular factors to regulate oligodendrocyte development.

Transcriptional Regulation of the Glutamate Receptor Gene Encoding the KA2 Subunit. The gene *GRIK5* encodes the rat kainate receptor subunit KA2. We have previously shown that 2 kb of 5′ flanking sequence of the *GRIK5* gene is GC-rich, TATA-less, can initiate transcription from multiple sites, and is sufficient to drive tissue-specific expression of a chimeric reporter gene in transgenic mice. By using DNAse I footprinting assays and electrophoretic mobility shift and (EMSA) supershift assays, we found that, of four consensus sequences present within 900 bp of the primary transcription initiation site, two sites bind to SP1 (-172 and -293) present in nuclear protein extracts isolated from the glial cell line CG-4. Furthermore, AP-2 consensus site present at -198 is also bound by extract protein, but the protein is not AP-2. We are currently searching for further *cis*-acting promoter elements and assaying the functional contribution of those identified so far by transient transfection of binding site point mutation constructs and reporter gene assays.

We have previously demonstrated that a 500 base pair DNA fragment of intron 1 negatively regulates *GRIK5* transcription. This fragment displays features similar to those of a silencer. Recent analysis using DNAse I footprinting and gel mobility shift assays demonstrated that the DNA element responsible for silencing activity is contained in a 24 nucleotide sequence. Mutational analysis of the nuclear protein binding site within this sequence defined an 11 nucleotide DNA element (5′-AAGGTCAGAGG-3′) accounting for the negative activity. Gel mobility shift assays performed with nuclear extracts from the oligodendrocyte cell line CG-4 indicated that mutation of 10

nucleotides in this region completely prevents binding to the relevant protein(s). This mutation also abolishes the inhibitory effect of the intragenic element on *GRIK5* transcription, as determined by reporter gene analysis. Mutations of 1-3 nucleotides either result in a partial recovery in binding activity and reporter gene transcription, or do not produce any significant effect. Base substitutions upstream or downstream of this element do not affect nuclear protein binding or reporter gene transcription. Gel mobility shift analysis revealed that the DNA-binding activity is present at much higher levels in rat brain nuclear extracts than in heart, kidney or liver. In the brain, the relative levels of this activity are higher between embryonic day 16 and postnatal day 6, and drastically decline in the adult. Higher DNA-binding activity was found in glia than in neurons.

By using the one-hybrid system, we have cloned several cDNAs from a rat brain library corresponding to proteins that bind to the *GRIK5* intronic silencer. *In vitro* translation of these cloned inserts using reticulocyte lysate generates products of molecular weights between 40 and 60 kDa, which exhibit specific binding to a single copy of the *GRIK5* intronic element. Sequencing of the clones revealed identity with several members of nuclear orphan receptor family, namely COUP-TFI, COUP-TFIII and NURR1. EMSA of postnatal day 2 rat brain extracts showed that formation of the shifted complexes can be specifically and preferentially competitively inhibited by synthetic oligonucleotides bearing consensus direct repeats (DR) of AGGTCA, separated by spacings of up to several nucleotides, with binding affinities whose rank order is DR1 > DR2 > DR3,4,5 > DR0. Partial competition also occurs with naturally-occurring elements such as the oxytocin estrogen-responsive element and β-retinoic acid response element, which is characteristic of promiscuous COUP-TF binding to diverse steroid- and retinoid-responsive elements. The presence of COUP-TFs and NURR1 in the postnatal brain extract complex was demonstrated in EMSA supershift experiments using specific anti-COUP-TF and NURR1 antibodies. Future experiments will analyze the role of these orphan nuclear receptors in the regulation of *GRIK5* transcription *in vivo*.

CELL BIOLOGY OF SECOND MESSENGER SYSTEMS

The Section on Neuronal Secretory Systems, led by ***James Russell***, studies the cellular processes that control glial cell signaling in response to neuronal activity. This focus encompasses investigation of processes that control intracellular calcium metabolism and calcium signaling in neuronal and glial cells and cellular mechanisms of signal transduction for receptors involved in calcium-based excitability. We are also involved in research to understand the regulation of excitability of mammalian pineal cells in their circadian control of melatonin secretion.

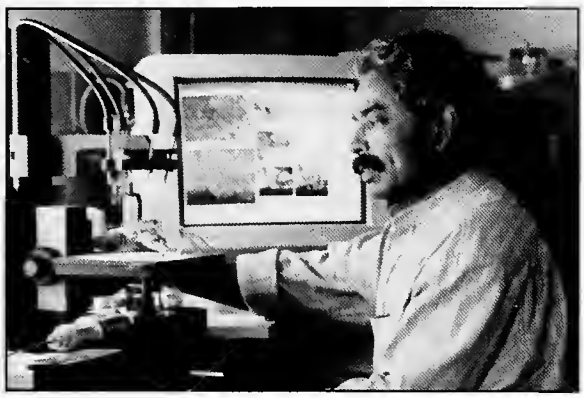
Intracellular Calcium Waves in Oligodendroglial Cells. It is now clear that glial cells monitor neuronal activity and respond to neuronal signals with a form of calcium-based excitability. Neither the cellular mechanism of initiation and propagation of astrocytic calcium waves nor the precise physiological function of such signaling in the brain is fully understood. During development, such signaling is thought to provide, through trophic substances whose release by glial cells is regulated by cellular activity, developmental cues for cell migration and path finding.

Previous work has described in detail the phenomenon of saltatory wave propagation in astrocytes following stimulation with neurotransmitters. Glial cells of the oligodendrocyte lineage in culture are better suited for this analysis because their long slender processes allow for treatment of cells as one dimensional entities. Agonist-evoked calcium waves were recorded in single cells which were then fixed on the microscope stage for immuno-cytochemical analysis of endoplasmic membrane proteins involved in calcium signaling and homeostasis as well as of organelle-specific indicators that specifically mark endoplasmic reticulum membranes and mitochondria. We hypothesized that high-density distribution of signaling proteins might be one of the specializations in wave amplification sites. Initially, we characterized the distribution of the different subtypes of $InsP_3Rs$. All three subtypes were found throughout the endoplasmic reticulum; however, there are differences in the intensity of labeling by the antibodies in different regions of the cells. We found that wave amplification sites are associated with significantly high-density distribution of $InsP_3R2$. Ample staining against $InsP_3R2$, however, was also observed in regions where the calcium release kinetics are several fold lower than at the wave amplification sites. This observation suggests that the distribution of the ER calcium release channels alone cannot explain the specialized calcium release at wave amplification sites.

Receptors, Channels, and Pumps. Astrocytes express ryanodine receptors. We found that activation of ryanodine receptors in both astrocytes and oligodendrocytes results in calcium waves. Furthermore, comparison of $InsP_3R$-mediated calcium waves and ryanodine receptor-evoked waves in the same cells shows that, in both cases, the wave amplification sites are identical. These results are consistent with the idea that glial cells possess a single functional calcium pool, which is specialized in certain regions of the cell that show wave amplification. While

it is possible that $InsP_3Rs$ and ryanodine receptors are colocalized at these specialized sites, an alternative or complementary explanation would be that the major factor (or factors) governing discrete calcium release sites is related to cellular characteristics other than the density of ER receptor channels alone.

Similar to the enrichment of $InsP_3Rs$ and ryanodine receptors at wave amplification sites, we also found SERCA pumps at these sites in high density. Immunocytochemistry shows high density patches of SERCA staining at sites of enhanced calcium release, and quantitative comparison using cross-correlation analysis confirmed this finding. We then compared the distribution of the intraluminal ER protein calreticulin with the distribution of wave amplification sites. Calreticulin belongs to the family of luminal proteins that bind to calcium ions with both low and high affinities and are believed to be involved in both buffering intraluminal calcium and in regulating calcium release via $InsP_3Rs$. Wave amplification sites in oligodendrocyte processes as well as in astrocytes reveal a bead-like concentration of calreticulin in the ER lumen. These findings suggested that multiple specializations may exist in wave amplification sites in glial cells.



James Russell

The cyanine dyes that we use to stain ER membranes also stain mitochondria, and we realized that mitochondria are always found at wave amplification sites. This discovery prompted careful analysis both of the distribution of mitochondria and their functional involvement in wave propagation in glial cells. Using the highly specific mitochondrial dyes, mitotracker and JC-1, we showed that, in oligodendrocyte processes, mitochondria are only found in wave amplification sites. Furthermore, mitochondrial poisons that uncouple oxidative phosphorylation (ATP synthesis) from respiration, such as protonophores and inhibitors of electron transport, markedly alter wave propagation, suggesting that mitochondria are essential for calcium signaling and wave propagation.

In summary, these experiments show that, in glial cells, regenerative propagation of agonist-evoked calcium waves is accomplished by enhanced calcium release at specific discrete wave amplification sites. These sites are approximately 1 to 2 μm in diameter and are found 5 to 7 μm apart. At these sites, calcium release kinetics are three- to five-fold higher than in the regions separating them, and may represent unitary calcium release sites. These wave amplification sites are characterized by multiple cellular specializations, which include the presence of one or more mitochondria, high-density distribution of $InsP_3Rs$, and high-density patches of SERCA pumps on the ER membrane, as well as bead-like concentrations of calreticulin in the ER lumen.

Pineal Cell Excitability. In the mammalian pineal gland, circadian rhythm in melatonin secretion is regulated by noradrenergic and neuropeptide inputs via cAMP and calcium-dependent mechanisms. We had previously found that, in pineal cells, VIP receptors are coupled to a cyclic GMP-gated cation channel, which results in increased cellular excitability upon activation. We discovered a large conductance (180 pS), voltage-dependent, non-selective cation channel on pineal cells with a novel mode of modulation by cAMP. PACAP, norepinephrine, or 8BrcAMP cause a 10 fold increase in the open probability of the channel, with a shift in voltage dependence towards zero from highly depolarized membrane potentials. The increase in open probability is followed by a change in current rectification properties, such that the channel is transformed from being inactive at rest to an inwardly rectifying cation conductance, when activated by norepinephrine or PACAP, which depolarize the cell. This channel is calcium-insensitive, blocked by cesium, and shows a permeability sequence: $K^+ > Na^+ > Li^+ > NH_4^+$.

Recently, another calcium-sensitive K^+ channel that is profoundly modulated by cyclic AMP and norepinephrine has been characterized. Voltage dependence, ion selectivity and open and closed time constants of this channel have been analyzed. Biophysical characterization of this channel is in progress, which will be followed by studies on its involvement in melatonin secretion. These studies are aimed at understanding the cellular mechanisms that support long-lasting stimulation necessary for circadian control of melatonin secretion mediated by neurotransmitters such as norepinephrine, VIP, and PACAP.

LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY

Chief
Mark L. Mayer, Ph.D.

Principal Investigators
Vittorio Gallo, Ph.D.,
James T. Russell, D.V.M., Ph.D.

Tenure-Track Investigator
Chris McBain, Ph.D.

Post-Doctoral Fellows
Robert Bähring, Ph.D.
Derek Bowie, Ph.D.
Li-Jin Chew, Ph.D.
Changhai Cui, Ph.D.
Jing Du, MD, Ph.D.
Mark Fleck, Ph.D.
Cristina Ghianl, Ph.D.
Laurel Haak, Ph.D.
Yang Li, Ph.D.
Victor Panchenko, Ph.D.
Steve Scherer, Ph.D.
Katalin Toth, Ph.D.

Pre-Doctoral Fellow
Alexander Eisen
Aaron Lackey

Guest Researchers
Yutaka Hasegawa, Ph.D.
Soraya Smaili, Ph.D.
William Hayes, Ph.D.

Senior Technical Specialists
George Dold
Lynn Holtzclaw

ANNUAL BIBLIOGRAPHY

Bähring R, Bowie D, Benveniste MJ, Mayer ML. Permeation and block of rat GluR6 glutamate receptor channels by internal and external polyamines. J Physiol 1997;502:575-89.

Chew L-J, Fleck MW, Wright P, Scherer SE, Mayer ML, Gallo V. Growth factor-induced transcription of GluR1 increases functional AMPA receptor density in glial progenitor cells. J Neurosci 1997;17:227-40.

Huang F, Gallo V. Gene structure of the rat kainate receptor subunit KA2 and characterization of an intronic negative regulatory region. J Biol Chem 1997;272:8618-27.

Keros S, McBain CJ. Arachidonic acid inhibits transient potassium currents and broadens action potentials during electrographic seizures in hippocampal pyramidal and inhibitory interneurons. J Neurosci 1997;17:3476-87.

Knutson P, Ghiani C, Zhou JM, Gallo V, McBain C. K^+ channel expression and proliferation are regulated by intracellular sodium and membrane depolarization in oligodendrocyte progenitor cells. J Neurosci 1997;17:2669-82.

Lijam N, Paylor R, McDonald M, Crawley JN, Deng CX, Herrup KE, Stevens K, Maccaferri G, McBain CJ, Sussman DJ, Wynshaw-Boris A. Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1. Cell 1997;90:895-905.

Mayer, M.L. Kainate receptors find homes at synapses. Nature 1997;389:542-3.

McBain CJ, Maccaferri G. Synaptic plasticity in hippocampal interneurons? A commentary. Can J Physiol Pharmacol 1997;75:488-94.

Pende M, Fisher TL, Simpson PB, Russell JT, Blenis J, Gallo V. Neurotransmitter- and growth factor-induced CREB phosphorylation in glial cell progenitors: role of calcium ions, protein kinase C and mitogen-activated protein kinase/ribosomal S6 kinase pathway. J Neurosci 1997;17:1291-301.

Sekiguchi M, Fleck MW, Mayer ML, Takeo J, Chiba Y, Yamashita S, Wada KA. Novel allosteric potentiator of AMPA receptors: 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide (PEPA). J Neurosci 1997;17:5760-71.

Sheppard CA, Simpson PB, Sharp AH, Nucifora FC, Ross CA, Lange GD, Russell JT. Comparison of type 2 inositol 1,4,5-trisphosphate receptor distribution and subcellular Ca^{2+} release sites that support Ca^{2+} waves in cultured astrocytes. J Neurochem 1997;68:2317-27.

Simpson PB, Mehotra S, Lange GD, Russell JT. High density distribution of endoplasmic reticulum proteins and mitochondria at specialized Ca^{2+} release sites in oligodendrocyte processes. J Biol Chem 1997;272: 22654-61.

Simpson PB, Russell JT. Mitochondria support inositol 1,4,5-trisphosphate-mediated Ca^{2+} waves in cultured oligodendrocytes. J Biol Chem 1996;271:33493-501.

Simpson PB, Russell JT. Role of sarcoplasmic endoplasmic reticulum Ca^{2+} -ATPase in mediating Ca^{2+} waves and Ca^{2+} release microdomains in cultured glia. Biochem J 1997;325:239-47.

LABORATORY OF COMPARATIVE ETHOLOGY

Stephen J. Suomi, Ph.D., Chief

The Laboratory of Comparative Ethology (LCE) investigates behavioral, cognitive, social-emotional, and physiological development in humans and in nonhuman primates. Both genetic and environmental influences and their multiple interactions are studied, using a comparative approach to characterize the origins, ontogeny, and outcomes of various behavioral phenotypes. Results of experimental studies of biobehavioral development in nonhuman primates are compared with findings from long-term prospective investigations of human infants and their families, as well as with data obtained by neuroscience techniques. Longitudinal designs are employed in most major studies to address issues of developmental continuity vs. change, and in many of these investigations a variety of both behavioral and physiological measures, reflecting multiple levels of analysis, are collected concomitantly. A major emphasis is placed on characterizing and understanding normative patterns of development so that deviant patterns can be readily identified and their consequences evaluated with respect to established norms.

GENETIC AND ENVIRONMENTAL DETERMINANTS OF PRIMATE BEHAVIOR

The Comparative Behavioral Genetics Section, headed by ***Stephen Suomi***, investigates biological and behavioral development in selected nonhuman primate species by focusing on interactions between genetic and environmental factors that affect the course of individual developmental trajectories throughout the life-span.

Long-Term Effects of Rearing with Surrogate Parent. Several studies completed this year focused on characterizing individual differences in specific aspects of biobehavioral responsiveness in rhesus monkey infants and on identifying factors contributing to such differences. Nursery-reared infants placed in an unfamiliar physical environment with their inanimate attachment object when they were 37 days old exhibit three distinctive patterns of response: some are active throughout the session, using their surrogate as a "secure base" to extensively explore their new settings; others are mostly quiet, clinging to their surrogate, sucking their digits, or even sleeping through the session; a few infants react with obvious fear and anxious-like behavior, characterized by excessive vocalizations and self-directed activity. These subjects are being followed prospectively to evaluate how well their distinctive response patterns as infants predict subsequent biobehavioral developmental trajectories during their juvenile, adolescent, and early adult years. In addition, heritability analyses of the three distinctive response patterns are currently under way.

Serotonergic Correlates of Maternal Behavior. A second study compared the behavioral development of rhesus monkey infants raised by mothers with relatively high chronic cerebrospinal fluid (CSF) levels of 5-hydroxy-indoleacetic acid (5-HIAA), the primary central serotonin metabolite, with those whose mothers have relatively low chronic CSF 5-HIAA concentrations. Although both groups of mothers show comparable rates of punitive behavior toward their offspring, infants reared by mothers with high 5-HIAA concentrations become more independent and socialized with other group members during their second six months of life than do infants reared by mothers with chronically low CSF 5-HIAA concentrations. The latter infants spend more time in relatively immature interactions with their mothers during the very months when the species norm is to focus increasing attention on interactions with peers. Infants' CSF 5-HIAA concentrations at six months of age correlate significantly and positively with those of their mothers, and individual differences in these concentrations remain relatively stable thereafter. A follow-up study utilizing cross-fostering procedures is currently in progress in order to better characterize genetic and experiential contributions, and their interactions, to the infants' differing developmental trajectories.

Behavioral Effects of Supplementing Monkey Infant Formula with a Long-Chain Fatty Acid. A third study examined the biobehavioral consequences of adding a long-chain essential fatty acid to the standard formula diet of nursery-reared rhesus monkey neonates, which raised their serum levels to those characteristic of infants raised (and nursed) by their mothers. The standard nursery formula diet, like most commercially available human infant formula diets, is lacking in certain long-chain essential fatty acids, and monkey infants raised on this standard formula consistently have lower serum levels of these fatty acids than their mother-reared counterparts. Infants receiving this dietary supplement exhibit accelerated motor maturation and enhanced

orientation capabilities during their first month compared with infants fed the standard formula diet, and in subsequent months their emerging behavioral profiles more closely resemble those of mother-reared age mates than those of their nursery-reared counterparts receiving the standard formula diet.

Serotonin Metabolism and Social Behavior in Feral Rhesus Monkeys. Another set of studies continued a long-term investigation of the relationship between serotonergic functioning (as indexed by CSF 5-HIAA concentrations) and behaviors associated with basic aspects of rhesus monkey male social life in field settings. Previous studies carried out in collaboration with NIAAA and LABS at the Morgan Island (SC) field site showed that juvenile male rhesus monkeys with impulsive/aggressive behavioral tendencies (and concomitantly low CSF 5-HIAA concentrations) are disproportionately likely to be expelled prematurely (i.e., well before puberty) from their natal social troop, are unlikely to then join all-male "gangs" (the species norm for emigrating pubertal males) or established troops, but are instead likely to become solitary, and subsequently unlikely to survive past puberty, typically perishing within a year. Research completed this past year focused on those few early-emigrating, low 5-HIAA males who survived to reproductive age in order to determine if they might be opportunistic breeders, i.e., disproportionally contribute to the population gene pool. Behavioral observations, in fact, suggest exactly the opposite: early emigrating males are significantly less likely to engage in consort relationships with females (who tend to avoid extended interactions with them whenever possible) than are males who emigrate later (and who have higher 5-HIAA concentrations). Moreover, those few consort relationships that are established by early emigrating males tend to be shorter in duration, involve fewer mounting sequences and fewer mounts within sequence, and result in fewer inseminations (indexed by presence or absence of sperm plugs) than consorts involving later emigrating males. DNA fingerprint data are currently being analyzed to assess the relative reproductive success of these males, but if the behavioral data are any indication, it seems unlikely that early emigrating males have been contributing much to the Morgan Island rhesus monkey population gene pool.

Plasma Cortisol as an Index of Reactivity in Maternal Behavior. A study involving wild rhesus monkeys at the other LCE field site on Cayo Santiago (PR) focused on the relationship between individual differences among adult females in behavioral and physiological response to brief capture (for veterinary examination) and patterns of maternal behavior. Females exhibiting high and stable heart rates also tend to have relatively high plasma cortisol levels following capture, replicating previous field data collected from infants and juveniles, as well as from adolescent males. These "high-reactive" females exhibit less ventral-ventral and dorsal-ventral contact, and initiate higher rates of contact breaks with their infants, than do "low-reactive" females; they also tend to groom other adult females more and allow their infants to take greater responsibility for maintaining proximity than do their low-reactive counterparts. These data provide the first empirical evidence that maternal behavior patterns can be influenced by maternal temperament in wild-living groups of rhesus monkeys.

Longitudinal Study of Rhesus Behavioral Profiles. Another set of analyses focused on longitudinal comparisons between the adult rhesus monkeys living in the five-acre outdoor enclosure at the National Institutes of Health Animal Canter (NIHAC), Poolesville, and a group maintained in indoor pens at the University of Massachusetts, whose founding members had similar rearing histories to those of the founding members of the field station colony but who had been maintained indoors since birth. These analyses revealed no basic qualitative differences (except for foraging behavior) and surprisingly few quantitative differences in behavioral profiles among young and middle-aged adults living in the two different environments. Follow-up comparisons of the founding members of both groups, now 24 to 27 years of age, revealed that aging is not associated with a general decline in activity levels across all behaviors but rather with a redistribution of how the aged monkeys apportion their different patterns of activity, including increases in social grooming behavior and decreases in locomotion and agonistic activities. Moreover, there is striking continuity in both group and gender differences, as well as in the stability of individual differences within each group, which were already evident when these elderly monkeys were young and middle-aged adults. Finally, measures of biobehavioral reactivity revealed remarkable stability of individual differences among both groups dating back to the monkeys' first year of life, suggesting that such differential patterns of response to environmental novelty and challenge remain relatively stable throughout the life span.

Studies of Capuchin Biobehavioral Development. Continued long-term study of capuchin monkey biobehavior development yielded several interesting findings regarding both species-normative patterns and individual differences in response to environmental challenge. Longitudinal analyses revealed that infant state measures collected during the first 11 weeks of life are significantly correlated with home cage behavior during months 2-6, in that individuals who were more active in early infancy subsequently spend more time alone, in

exploration, and in active play later on, and less time in contact with their mother, than do individuals who were more quiet as infants. In addition, infants who were more often asleep on mothers, or inactive when awake, subsequently explore less and tend to be more agitated when briefly separated from their group. These quieter infants also subsequently exhibit higher cortisol levels during brief separation at 1 year of age. Infants who were awake and active more early in life, and away from mothers more, show less stereotypy, more exploration of their environment, and lower cortisol levels during a comparable separation.

Tool Use in Capuchins. Research investigating the tool-using capacities of capuchin monkeys was continued during the past year, with special emphasis on studies utilizing an experimental paradigm that enables groups of capuchins housed in adjacent pens to obtain and share highly desirable food items only if tools needed to obtain the food items are also shared. Several monkeys in both groups mastered this complex tool- and food-sharing task, providing the first evidence of tool-sharing in any primate species other than humans and chimpanzees. The degree to which laterality (handedness) is involved in various tool-using tasks, as well as in other activities, was also investigated in several studies. Most successful tool-using monkeys exhibit a significant right-handed bias in their manipulation of tools, and this bias tends to be stronger in adults than in juveniles. The right-handed bias is more pronounced in tasks requiring a bipedal posture than in those that can be solved while sitting or maintaining a quadrupedal posture. Moreover, the monkeys displaying the strongest lateral bias in tool-using tasks also display the strongest lateral bias in eye dominance and rotational behavior, and they are also the individuals most likely to use a mirror to guide manipulation of objects hidden from direct view.

Stephen Suomi with a collaborator

Another set of analyses of tool-use by capuchin monkeys identified factors contributing to the dramatic individual differences in tool-using proficiency consistently reported in the literature (and clearly evident in the LCE capuchin colony as well). Detailed study of the emergence of tool-use in juvenile capuchin monkeys revealed that individual differences in initial tool-use activity are related to previous differences in object manipulation styles. These differences in manipulation style are, in turn, related to adrenocortical responsiveness during separation. Juveniles exhibiting high cortisol levels during separation are subsequently least likely to engage in extended complex manipulation of objects that potentially could be used as tools. Other analyses revealed that individuals that are first exposed to tools after the age of ten are significantly less likely to develop tool-using skills than individuals whose are first exposed to tools before the age of ten. On the other hand, few monkeys under two years of age (and no individuals younger than eight months) exhibit tool-using capabilities. Thus, it appears that there is a critical or sensitive period for acquisition of tool-using capabilities. In addition, certain early social experiences may also be crucial determinants of subsequent successful tool-use. In particular, juvenile capuchin monkeys whose mothers had died (in each case, of natural causes) prior to their third birthday failed to develop tool-using skills, even when given considerable exposure to tools and tool-using situations, whereas virtually all youngsters whose mothers remained in their social group until at least their third birthday eventually developed such skills. These findings suggest that early disruption of the mother-infant relationship can hinder the development of the complex cognitive and motor skills required for successful tool-use, even in the face of considerable exposure to tool-using opportunities during this critical or sensitive developmental period.

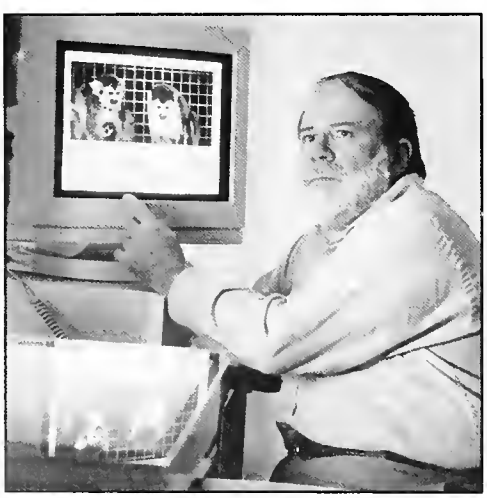
NEUROETHOLOGY OF PRIMATE AUDITORY COMMUNICATION

The Unit on Developmental Neuroethology Section (UDN), headed by ***John Newman***, uses neuroscience techniques to study brain mechanisms involved in the production of various types of primate vocalizations, to examine subtle acoustical differences in these characteristic calls, and to investigate their function in several primate species, including humans. Research carried out in the UDN during this past year continued to focus on vocalizations and other social behaviors and their associated brain processes in several primate species.

Vocal Development in Macaques. One set of analyses focused on vocal development over the first five months of life in mother-reared and nursery-reared rhesus monkey infants. Vocalizations were recorded during routine Brazelton assessments at one, two, three, and four weeks of age, as well as during brief separations at two, three, four, and five months. Each vocalization of suitable acoustic quality was digitized, then subjected to Fourier analysis; the resulting FFT files were then used to calculate a large number of acoustic parameters. Discriminant analysis of these parameters yielded five distinctive clusters of vocalizations, 77% of which were subsequently classified as one of two distinctive "coo" call types (the other three call types include complex screams, noisy calls with generally negative frequency trends in the spectral energy, and short, low-frequency calls, respectively). Subsequent analyses of the "coo" call data indicated that both types of "coo" calls become more stable, the distinctive call structures become more regular, and the overall pitch drops with increasing age; in addition, the relative proportion of each "coo" type changes somewhat over time. In general, nursery-reared males tend to emit one "coo" type more frequently than mother-reared males or females from either rearing condition. Gender differences in specific "coo" call parameters are evident at each age of recording, whereas rearing condition differences are apparent only at the earlier ages. Finally, clear-cut individual differences in "coo" call parameters are apparent as early as one week of age and continue through the first five months, although the underlying reasons for the individual differences at the early ages differ from those at the older ages. Specifically, the calls at one to two weeks tend to be highly variable, but the nature of the variability is individually distinct, whereas by four to five months the calls of all individuals are much more stereotyped and exhibited differences in basic acoustic parameters.

Heart rate data were collected via telemetry from each infant during the vocal recording sessions at three, four, and five months, and a blood sample was also obtained at the end of each session. In general, heart rates decrease significantly over each 30 minute recording session, as do scream vocalizations, whereas "coos" tend to increase throughout each session. Linear regression analyses revealed significantly positive correlations between mean heart rate and total vocal activity at three months, but not at the later ages; this relationship is especially strong for "coo" calls, but not for the other vocal categories. No significant relationships were found between plasma cortisol levels and any of the vocal parameters.

Vocal Development in Marmoset Monkeys. Another set of studies investigated the ontogeny of separation "phee" call use and structure in common marmosets. Marmosets of all ages reliably produce this species-specific type of "phee" call when briefly separated from their familiar social group, but there are gender- and context-specific features of the call that are not present in infancy; on the other hand, previous work in this lab has also identified acoustic features of infant "phee" calls that appear to disappear during development. "Phee" calls were recorded from infants shortly after weaning (age three months), shortly after the birth of a new sibling (age six months), and again at the approximate age of puberty onset (age twelve months); analyses completed this past year focused on the six- and twelve-month data. The total rate of separation "phee" calling declines from six to twelve months, as does the number of one-syllable calls, whereas that of two-syllable calls increases over the same period. There are no significant gender differences in "phee" call usage at either age.



John Newman

These juvenile marmosets also show developmental changes in the acoustic structure of their "phee" calls, with significant increases in peak frequency, frequency syllable range, and some duration features from six to twelve months of age. Discriminant analyses revealed significant gender differences in call structure at both ages. Additional analyses characterized clear-cut individual differences in "phee" call structure independent of gender at 6 months, and these differences remained stable and robust in the 12 month tests. We continued our investigations of the developmental aspects of contextual influences on "phee"-calling in postpubescent marmosets. Previous work had shown that the characteristic adult form of territorial "phee" call is suppressed in adolescent and nonbreeding adult members of established social groups, but that the onset of adult-like call production is rapid after pairing with a new partner away from the group. Discriminant analyses completed this past year demonstrated that the increase in "phee" call production is largely the result of an increase in territorial, rather than separation, "phee" call

subtypes. In contrast, individually housed postpubescent marmosets tend to emit separation, rather than territorial, "phee" calls, and the rate of such calling increases when they are moved into unfamiliar settings. These findings support the idea that territorial "phee" calling is situationally dependent and further suggest that marmosets can establish social relationships with neighboring individuals even in the absence of opportunities for extended mutual physical interaction.

Studies of the Molecular Basis of Parenting in Marmosets. The extensive family interactions of marmosets are characterized by not only specific vocal patterns but also by ubiquitous caretaking and affiliative behaviors seen in all noninfant family members. Previous work in this and other laboratories has suggested major roles for the neuropeptides oxytocin and vasopressin in mediating many of these contact-oriented behaviors. Investigations completed this year utilized *in situ* hybridization, immunocytochemical, and receptor audiographic techniques to map vasopressin brain pathways in adult marmosets. Clusters of cells labeled for arginine vasopressin (AVP) mRNA or stained for AVP immunoreactivity were found in the paraventricular, supraoptic, and suprachiasmatic nuclei of the hypothalamus. Scattered AVP-producing cells were also found in the lateral hypothalamus and the bed nucleus of the *stria terminalis*. Neither AVP mRNA labeling nor AVP immunoreactivity was detected in the amygdala. Although AVP-immunoreactive fibers were evident outside the hypothalamic-neurohypophyseal tract, a plexus of fibers in the lateral septum was not detected, in contrast to the case with rodents. Receptor autoradiography revealed specific binding to AVP receptors in the *nucleus acumbens*, diagonal band of Broca, lateral septum, bed nucleus of the *stria terminalis*, suprachiasmatic nucleus, paraventricular nucleus, amygdala, and anterodorsal and ventromedial hypothalamus. Together, these data provide a comprehensive picture of AVP pathways in the marmoset brain, demonstrating differences from rodents in the distribution of cell bodies, fibers, and receptors.

CHILDREN'S MENTAL AND SOCIAL DEVELOPMENT

The Child and Family Research Section (CFRS), headed by ***Marc Bornstein***, examines cognitive, language, and social-emotional development in human infants and children, with special emphasis on the relations among early attentional processes, social stimulation from and interactions with care-givers, and subsequent cognitive and behavioral capabilities. Research carried out in the CFRS this past year continued a series of longitudinal investigations into the development of attentional and cognitive capabilities, and of personality characteristics, into the influences of various aspects of maternal care, and into the emergence of play and language and their relationship to representational capabilities in human infants and children over the first four years of life. These investigations utilized both normative and clinical samples of children and their mothers from a variety of sites both within the U.S. and abroad. Standardized assessments of child capabilities and maternal characteristics at common age points (e.g., 5, 20, and 48 months) in most of the samples have permitted direct comparisons of various aspects of child development both within and between a variety of distinctive cultures.

Marc Bornstein

Development of Representational Abilities in Children. Several sets of analyses completed this past year focused on normative aspects of attentional, language, play, and representational development. In one study of early attentional patterns, infants in longitudinal and cross-sectional samples were habituated to three different visual stimuli and given post-habituation tests on day one and at one, two, three, and four months of age. We found consistent differences among the three stimuli in habituation times, which are maintained at all ages, while for two of the three stimuli there is a marked increase in habituation times at two months of age. We also found changes in post-habituation preferences across age for two of the stimuli. These findings clearly indicate that visual perceptual development is characterized by both continuity and change throughout early infancy.

Another short-term prospective longitudinal study of young infants examined specific predictive relations between mothers' responsiveness to their five-month-olds' nondistress activities and to their vocal distress behavior and the infants' attention span, symbolic play, and language comprehension at 13 months. Maternal

responsiveness to infant nondistress activities, but not to infant distress, at five months uniquely predicts infant attention span and symbolic play, but not language comprehension, at 13 months. Mothers' responsiveness at 13 months is only marginally positively associated with these 13-month infant abilities. These findings support the view that the effects of maternal responsiveness on infant mental development are specific but indirect, rather than generic and direct, during this part of infancy.

Effects of Parenting in Child Development. A related study examined covariation among specific maternal behaviors and their differential prediction of children's language comprehension across the transition to beginning speech. Videotapes of mother-infant dyads during free play in their homes when the children were 9 and 13 months old were scored for two factors characterizing maternal interaction, verbal sensitivity and verbal intrusiveness; these factors were found to be stable across the 9- and 13-month taping sessions. Nine-month maternal sensitivity, but not intrusiveness, uniquely predicts 13-month child language comprehension over and above the child's 9-month scores, which are themselves predictive of 13-month performance. Maternal verbal sensitivity is especially influential in promoting comprehension among children who are initially lower in language comprehension, a finding that has significant implications for the design of intervention strategies. These and other recent findings suggest that maternal responsiveness is profitably categorized into subtypes that relate to different domains of child outcomes in specialized ways.

Development of Symbolic Thought in Children. The association between children's language development and other aspects of thinking that require symbolic functioning was examined in older children by measuring their ability to comprehend actions with imagined objects at 24, 36, and 48 months of age. We found that measures of symbolic gesture ability at the three ages are all positively related, but only the correlation between 36- and 48-month gesture comprehension reaches statistical significance. Significant concurrent associations were found between language and gesture comprehension at 24 and 36 months, respectively, even after 48-month IQ was partialed out. This pattern of results points to an underlying common factor in language and gesture that is separate from intellectual capacity, as assessed by traditional IQ measures.

Cross-Cultural Studies of Representation in Children. Symbolic and other aspects of children's play were further investigated in a cross-cultural study in which Argentine and U.S. children and their mothers were compared on dimensions of play and interaction when the children were 20 months old. Analyses revealed notable cultural differences: Argentine children are more advanced in symbolic play than U.S. children, and Argentine mothers are more engaged in diverse play activities than U.S. mothers, as well as being more socially available to children. Advanced play by Argentine mothers focuses on promoting "other-directed" acts of pretense, social play, and verbal praise. Generally speaking, girls engage in more symbolic play than boys, and mothers of girls also engage in more symbolic play than mothers of boys. In both cultures, individual variation in mothers' symbolic play, but not social play, is specifically associated with individual variation in children's symbolic play. These findings suggest that during an early period of symbolic and social development, as expressed through diverse forms of play, Argentine and U.S. dyads emphasize different modes of representation and interaction, perhaps reflecting larger cultural concerns for collectivism vs. individualism.

Knowledge of Parenting. A different set of analyses revealed that mothers who are knowledgeable about the development of child play provide their children with appropriately challenging play interactions. In this study, mothers of children ranging in age from 6 to 58 months were tested for their knowledge about play and language development, using a paired-comparisons procedure in which they were asked to determine, for pairings of play and language items, respectively, which item was the more advanced developmentally. This procedure was repeated within a two-week period so that the short-term stability of mothers' knowledge could also be assessed. Play items included exploratory behaviors, nonsymbolic acts, and symbolic acts. Language items included prelinguistic communications, single-word utterances, and multi-word utterances of varying sophistication. In general, mothers' orderings of play and language items match those established in the developmental literature and are stable over the short term. Mothers' knowledge about language development tends to be stronger than and unrelated to their knowledge about play, suggesting that maternal knowledge about developmental domains is specific. We also found that mothers' judgments about the developmental timing of both play and language depend on their children's current developmental stage, in that mothers are less accurate at estimating the timing of milestones that their children have mastered many months earlier, supporting the notion that mothers' sensitivity to play and language development is largely informed by their children's recent rather than past achievements in these specific areas.

Behavioral Pediatrics. Other studies completed in this year addressed the influence of emotion and previous experience on children's appraisals of the value and efficacy of strategies for coping with stressful medical

situations. Relations among children's trait anxiety, history of previous hospitalization, orientation toward or away from information about threat, and appraisals of strategy value and efficacy were evaluated, controlling for age and gender. We found that children with higher levels of trait anxiety are more likely to appraise behavioral and cognitive distraction strategies as valuable. Children with lower levels of trait anxiety who have been hospitalized previously appraise information-approach strategies as less valuable than do all children with higher levels of anxiety and than children with lower levels of anxiety who have never been hospitalized. History of previous hospitalization also predicts children's appraisals of the efficacy of behavioral distraction strategies in changing unpleasant feelings associated with anticipated treatment, thus, children who have been hospitalized previously appraise these strategies more favorably. Orientation toward information about threat, as measured in this study, does not relate to children's appraisals of coping strategy value or efficacy. These results indicate that children's emotions and early hospitalization experiences influence processes of cognitive appraisal, which in turn are an integral facet of coping as a real-world problem-solving activity.

Perspectives on Parenting. Recent studies of parenting have begun to focus on parents' views about their own parenting, information that has both descriptive and explanatory value. Two salient aspects of parental thinking are self-evaluations of parenting and attributions of successes and failures in parenting. In a multicultural study, information was obtained about mothers' self-evaluations of their competence, satisfaction, investment, and role balance in parenting and about mothers' attributions for their successes and failures across diverse parenting tasks. These self-evaluations and attributions were compared in samples of mothers in seven nations, Argentina, Belgium, France, Israel, Italy, Japan, and the United States. Some significant cross-cultural similarities emerged. Systematic country effects for both self-evaluations and attributions also emerged and were interpreted in terms of specific cultural proclivities and emphases. We found that child gender is never a systematic factor.

In another study, the influences of children's age, and parents' and children's gender, on parents' attributions and emotional and behavioral responses to their children's successful and unsuccessful social and academic outcomes were investigated in 76 dual-parent families (mothers and fathers) of fifth, eighth, and eleventh grade children. The results of this study suggested that, from fifth grade on at least, the ways parents explain the causes of, and respond to, their children's social behavior and academic outcomes involve complex interactions of children's age, children's gender, parents' gender, domain, and outcome.

SOCIAL AND EMOTIONAL DEVELOPMENT IN CHILDREN

Adaptation to Daycare in Infancy. Research carried out this past year in the Section on Social and Emotional Development, which is led by ***Michael Lamb***, centered on five major areas of developmental study. A new short-term longitudinal study investigated the transition to daycare in 11- to 18-month-old infants, using measures of heart rate and adrenocortical reactivity and attachment classification collected prior to and following initial enrollment, including days on which mothers stayed with their infants at the daycare center. Analyses of temperament measures identified four groups of infants, difficult, shy, easy, and active-expressive, with the clearest discrimination between the easy and difficult patterns. We found that easy infants spend the most time in interactions with the care providers on the last day on which their mother accompanied them. Difficult infants have more frequent discordant interactions with their care providers both before and after separation, and they are also less likely to engage in object-mediated interactions and more likely to engage in affectively toned interactions (i.e., those involving comforting or soothing) with their care providers. These results thus confirm that perceived temperament affects the infants' initial adaptation to daycare.

Fifteen weeks after enrollment in daycare, the majority (88%) of these infants showed signs of becoming attached to their care providers: 32% of the total appeared to be securely attached, while 56% appeared to have insecure and/or disorganized attachments. Security of attachment to care providers was not predicted by the security of infant-mother attachment either prior to daycare or after 9 weeks of daycare experience. However, we found that the security of care provider attachment, as assessed in the Strange Situation, is related to indices of the care providers' behavior, as well as the infants' own behavior in daycare. In particular, infants who develop secure attachments to their care providers often display negative emotions, and they are also more likely both to initiate interaction with their care providers and to accept physical comfort from them. Ongoing analyses are designed to explore the combined effects of attachment and temperament on the infants' adjustment to daycare. The physiological data have yet to be analyzed.

In an independent sample, toddlers were observed over the course of typical weekdays, and analyses revealed that measures of the amount of maternal vocalization and stimulation per unit of observed time are higher

among daycare children than home-only children. The daycare children also vocalized at home more than when they were in their daycare centers, where they appeared to have fewer opportunities for social interaction. Surprisingly, however, the total amounts of social interaction experienced by these toddlers over the course of the day did not differ, regardless of whether or not they spent time in daycare. These findings suggest that researchers concerned with the effects of daycare need to include more systematic assessments of children's actual experiences, both in and out of daycare settings.

Cultural Differences in Infants' Everyday Experiences. In a long-term study of Euro-American and Central-American mothers in the Washington area, mother-infant interaction during free play, teaching, and feeding situations at 4, 8, and 12 months of age were coded microanalytically. Individual differences on the discrete measures of infant and maternal behavior during bouts of mother-infant play and teaching were found to be neither stable nor consistently related to the later quality of infant-mother attachment, but measures of responsivity during play and mutual coordination in the teaching session are stable over time and are strongly related to the quality of attachment. In both groups, mothers of secure infants score around the middle on the measure of responsivity during play, whereas mothers of resistant infants score at either higher or intermediate levels. Mothers of avoidant infants tend to score lower in the Euro-American sample and higher in the Central American sample. Mutual coordination of maternal teaching is highest in dyads involving securely attached infants. Didactic maternal behavior in the teaching situation decreases as the infants grow older, whereas task-oriented infant behavior increases with age. Individual differences in maternal teaching behavior are not stable over time, although the level of maternal vocalization is. There are only minor differences between the samples with respect to levels of mutual coordination or the probability of secure attachment behavior in a standardized Strange Situation assessment. Dyads who were later classified as disorganized had negative scores on a measure of joint attention to objects.


Michael Lamb

Other analyses focused on the general patterns of everyday experiences in these families when the infants are three months old. Descriptive analyses revealed remarkable similarities in the everyday experiences of infants in the two diverse groups. Subsequent fine-grained analyses revealed no group differences in the amounts of time spent by the mothers and infants in well-attuned states, or in different functional contexts, but the Euro-American dyads spend more time in disharmonious states. Securely attached dyads in both samples spend more time in well-attuned states, as well as in playful interactions, rather than not interacting. Forty-five-minute blocks of observation yield highly unreliable measures of individual differences, but reliability increases considerably as the duration of the observations is expanded. The amounts of time spent in well-attuned and in disharmonious interactions varies substantially across contexts, with notable differences between the two groups. The results indicate that, from a strictly methodological standpoint, one can maximize subcultural differences by focusing on single contexts, and minimize subcultural differences by averaging across a variety of naturally occurring contexts when collecting such interactional data.

Long-Term Effects of Contrasting Early Experiences. Another major area of study in the SSED is our continuing long-term follow-up investigation of a Swedish sample of children who have been raised since 16 months of age in a center-based daycare setting, in a home-based daycare setting, or at home with one or both parents. Previous analyses of the longitudinal data collected on these children from toddlerhood through entry into the first grade consistently demonstrated that while the type of child care has little or no differential impact on any measures, the quality of home care and quality of alternative care has powerful effects on emerging social skills, personal maturity, and behavior in compliance-eliciting settings. During the past year, the association between child-care arrangements and subsequent cognitive and personality development was examined. Parental ratings of field independence, ego resilience, and ego-control obtained with the California Q-set when the children averaged 28, 40, 80, and 101 months of age revealed virtually no differences between the developmental trajectories of the children in home care and in center-based daycare. In contrast, ego-undercontrol decreases less, while ego-resilience and field independence increase less, in children who attend family daycare than in

the children in either center care or exclusive parental care. Surprisingly, the quality of home and out-of-home care, as well as the socioeconomic status and family background, does not appear to qualify these long-term effects of the type of care experienced.

The divergent developmental trajectories of the children in the home-care and the center-based daycare groups differ with respect to the development of verbal abilities as well. Tested ability in second grade is related to the number of months children have spent in center-based day care before 3½ years of age. Structural and dynamic aspects of the child care quality also predict cognitive abilities among those children who have spent at least 36 months of their preschool months in out-of-home care. Both tested and rated cognitive abilities in second grade are related to earlier measures of verbal ability and to paternal involvement during the pre-school years.

Another series of analyses focused on the fathers' relative involvement in the children's lives. Parents provided time-diary estimates of paternal participation and independent estimates of the mother's and father's responsibility and their children's preferences when the children were 16, 28, 40, 80, and 102 months of age. Children reported on their preferences and their parents' responsibilities in the last two phases. Analyses showed convergence between parents' and children's estimates of paternal involvement and parental preferences, underscoring the reliability of the reports. Though poorly correlated with one another, relative (i.e., extent of paternal participation relative to that of the mother) and absolute (i.e., estimates of the amount of time devoted to specified activities) measures of paternal involvement were found to be modestly stable over time, with higher stability evident on reports of relative rather than absolute levels of involvement.

Family Origins of Aggression Among Boys. In a series of studies of childhood attributions and aggression, we found that both maternal and child attributions, as revealed in interviews about one another, are significantly related to the coerciveness of their interactions exhibited in two game-like tasks. The most aggressive dyads are those in which both mothers and sons perceive hostile intent in the other. Mothers' (but not sons') attributions are also related to teachers' reports of the children's aggression in the classroom. Boys who report many stressful events in their lives also tend to behave coercively with their mothers and are viewed by their teachers as more aggressive and less socially competent with peers. The relation between stressful life events and the boys' reported aggression with their peers is mediated, in part, by the boys' coerciveness with their mothers. The association between boys' coerciveness with their mothers and social acceptance by peers appears to be mediated by the aggressiveness of the reported interactions with peers. Longitudinal analyses suggest that the boys' earlier aggression is not predictive of more negative subsequent attributions in mothers, whereas mothers' negative behavior indeed predicts subsequent negative biases on the part of their sons. However, even after considering both mothers' and children's earlier negative behavior, children's negative attributions about their mothers contribute significantly to the aggressiveness of their subsequent behavior with their mothers. The same is not true for mothers: mothers' earlier attributions indirectly influence their subsequent aggressive behavior with their sons.

Enhancing the Quality and Amount of Information Provided by Alleged Victims of Child Abuse. A series of studies carried out in the SSED this past year continued earlier efforts to evaluate and improve upon the Criterion-Based Content Analysis (CBCA) system for eliciting and assessing statements by children alleged to have been victims or witnesses of sexual abuse. Transcripts of forensic interviews with alleged victims of child sexual abuse were scored for the presence or absence of the CBCA criteria believed to be more characteristic of accounts of experienced rather than nonexperienced events. As predicted, more of the CBCA criteria are present in accounts independently rated as "likely" or "very likely" to have occurred than in accounts of events deemed unlikely or very unlikely to have occurred. Several of the criteria were found to be especially helpful in distinguishing plausible from implausible accounts, but the group differences are not as dramatic as those reported in earlier studies.

In several subsequent studies carried out in Israel and the U.S., we were able to confirm the prediction that open-ended invitations yield significantly longer and more detailed responses than directive, leading, or suggestive utterances, regardless of age and culture. The superiority of invitations appears greater when children report experiencing three or more, rather than only one, incidents of abuse. Invitations are rarely used, however, and investigators often fail to elicit more information from children who reported multiple incidents of abuse than from children who report only one incident. Others studies showed that anatomically detailed dolls do not facilitate the acquisition of information from children. Younger children provide briefer and less detailed responses, however.

In an experimental field study, we showed that a script containing many open-ended utterances to establish rapport elicits much more detailed accounts from alleged victims than does a script involving many direct questions. Children who have been trained in the open-ended condition actually provide 2½ times as many details and words in response to the first substantive utterance as do children in the direct introduction condition. Ongoing research is designed to determine whether (1) the additional details are accurate, and (2) yet richer and more useful accounts can be obtained if the entire interview is scripted. Systematic attempts are also being made to explore various enhancement techniques, such as context reinstatement. In a lab analog study, we explored whether the timing of the presentation of misleading postevent information affects the incorporation of this information into children's reports. Four-year-olds participated in a staged event, dressing up in costumes and having their photographs taken. An unfamiliar adult then spoke to the children about the event either a day (immediate condition) or a month (delayed condition) later and provided misleading information about the event. When questioned five weeks after the event, children in the delayed condition included more of the misleading details in their responses than did children in the immediate condition. The accuracy of the details recalled, however, was not affected by the timing of the misinformation.

LABORATORY OF COMPARATIVE ETHOLOGY

Chief
Stephen J. Suomi, Ph.D.

Principal Investigators
Marc H. Bornstein, Ph.D.
Michael E. Lamb, Ph.D.
John D. Newman, Ph.D.

Staff Scientists
Maurice Haynes, Ph.D.
Kathleen Sternberg, Ph.D.

Staff Veterinarian
Mark Haines, D.V.M.

Senior Fellows
Lieselotte Ahnert, Ph.D.
Maribeth Champoux, Ph.D.
Kathlyn Rasmussen, Ph.D.

Senior Technical Specialist
Peggy O'Neill Wagner

Pre-Doctoral Fellows
Wendy Airoso
Lauren Alistair
Katherine Choe
Angele Fauchier
Lynn Havsy
Tara Herzberg
Leslie Kim
Heather Kuhn
Valerie Lewis
Steve Lindell
Samantha Litzinger
Chanya Liv
Gaby Maimon
Molly McMillan
Courtney Shannon-Lindell
Rachel Sheldrick
Darlene Smucne
Heather Stec

Post-Doctoral Fellows
Carol Andreassen, Ph.D.
Cecile Bassen, M.D.
Linda Cote, Ph.D.
Janice Genevro, Ph.D.
Birgit Leyendecker, Ph.D.
Janet Norcross, Ph.D.
Samia Dawud Noursi, Ph.D.
Yael Orbach, Ph.D.
Kim Roberts, Ph.D.
Lucy Roberts, Ph.D.
Angelika Timme, Ph.D.
Gregory Westergaard, Ph.D.

Guest Researchers
Shashi Bali, M.D.
Olga Bazhenova, Ph.D.
Gayle Byrne, Ph.D.
Douglas Flor, Ph.D.
Irit Hershkowitz, Ph.D.
Dee Higley, Ph.D.
Philip Hwang, Ph.D.
Melinda Novak, Ph.D.
Eric Phoebus, Ph.D.
Steven Porges, Ph.D.
Andra Rose, Ph.D.
Susan Theut, M.D.

ANNUAL BIBLIOGRAPHY

Berman CM, Rasmussen KLR, Suomi SJ. Group size, infant development, and social networks: a natural experiment with free-ranging rhesus monkeys. Animal Behav 1997;53:405-21.

Bornstein MH. Nutrition and development: Observations and implications. In: Dobbing J, ed. Developing brain and behaviour: the role of lipids in infant formula. London: Academic Press 1997;475-509.

Bornstein MH, Slater A, Brown E, Roberts E, Barrett J. Stability of mental development from infancy to later childhood: three "waves" of research. In: Brenner G, Slater A, Butterworth G, eds. Infant development: recent advances. Hove, England: Psychology Press 1997;191-215.

Broberg AG, Weßels H, Lamb ME, Hwang CP. The effects of day care on the development of cognitive abilities in eight-year-olds: A longitudinal study. Develop Psychol 1997;33:62-69.

Champoux M, Higley JD, Suomi SJ. Behavioral and physiolgical characteristics of Indian and Chinese-Indian hybrid rhesus macaque infants. Develop Psychobiol 1997;31:49-63.

Champoux M, Kriete MF, Eckhaus MA, Suomi SJ. Behavioral and physical concomitants of congenital hydrocephalus in a rhesus macaque infant. Lab Animal Sci 1997;36:56-61.

Fracasso MP, Lamb ME, Scholmerich A, Leyendecker B. The ecology of mother-infant interaction in Euro-American and immigrant Central American families living in the United States. Internat J Behav Develop 1997;20:207-17.

Higley JD, Suomi SJ, Linnoila M. Excessive alcohol consumption, inapprorpriate aggression, and serotonin: a nonhuman model of alcohol abuse. Rec Dev Alcohol 1997;13:191-219.

Horowitz SW, Lamb ME, Esplin PW, Boychuk TD, Krispin O, Reiter-Lavery L. Reliability of criteria-based content analysis of child witness statements. Legal Criminological Psychology 1997;2:11-21.

Lamb ME, ed. The role of the father in child development (third edition). New York: Wiley, 1997.

Lamb ME. The role of the father in child development: An introduction and overview. In: Lamb ME, ed. The role of the father in child development (third edition). New York: Wiley 1997:1-18, 309-13.

Lamb ME. The development of father-infant relationships. In: Lamb ME, ed. The role of the father in child development (third edition). New York: Wiley 1997:104-20, 332-42.

Lamb ME. Fathering in America: new challenges and champions. Contemp Psychol 1997;41:911.

Lamb ME, Billings LL. Fathers of children with special needs. In: Lamb ME, ed., The role of the father in child development (third edition). New York: Wiley 1997:179-190, 356-60.

Lamb ME, Hershkowitz I, Sternberg KJ, Boat B, Everson MD. Investigative interviews of alleged sexual abuse victims with and without anatomical dolls [see comments]. Child Abuse Negl 1996;20:1251-9.

Lamb ME, Sternberg KJ, Esplin PW, Hershkowitz I, Orbach Y. Assessing the credibility of children's allegations of sexual abuse: insights from recent research. Learn Indiv Diff 1997;9:175-94.

Lamb ME, Sternberg KJ, Esplin PW, Hershkowitz I, Orbach Y, Hovav M. Criterion-based content analysis: a field validation study. Child Abuse Neglect 1997;21:255-64.

Lamb ME, Sternberg KJ, Thompson RA. The effects of divorce and custory arrangements on children's behavior, development, and adjustment. Fam Conciliation Crt Rev 1997;35:393-404.

Leyendecker B, Lamb ME, Fracasso MP, Scholmeric A, Larson C. Playful interactions and the anticedents of attachment: a longitudinal study of Central American and Euro-American mothers and infants. Merrill-Palmer Quart 1998;43:24-47.

Leyendecker B, Lamb ME, Scholmerich A, Fricke D. Contexts as moderators of observed interactions: a study of Costa Rican mothers and infants from differing socioeconomic backgrounds. Internat J Behav Develop 1997;21:15-34.

Malkova L, Mishkin M, Suomi SJ. Comparisons of early vs. late medical temporal lobe lesions on socioemotional behavior in rhesus monkeys. Annal NY Acad Sci 1997;807:538-40.

Mayes, Lc, Bornstein MH. The development of children exposed to cocaine. In: Luthar SS, Burak AA, Cicchetti D, Weisz JR, eds. Developmental psychopathology: perspectives on adjustment, risk, and disorder. New York: Cambridge University Press, 1997;166-88.

Mehlman PT, Higley JD, Fernald BJ, Sallee FR, Suomi SJ, Linnoila M. CSF 5-HIAA, testosterone, and sociosexual behaviors in free-ranging male rhesus macaques in the mating season. Psychiatry Res 1997;72:89-102.

Newman JD, Bachevalier J. Neonatal ablations of the amygdala and inferior temporal cortx in rhesus macaques alter vocal response to social separation during the first year of life. Brain Res 1997;758:180-6.

Newman JD, Berhards DE. The affiliative vocal subsystem of squirrel monkeys. In: Carter CS, Kirkpatric B, Lederhendler I eds. The integrative neurobiology of affiliation. Ann NY Acad Sci 1997;807:546-8.

Norcross JL, Newman JD. Social context affects phee call production by nonreproductive common marmosets (Callithrix jacchus). Am J Primatol, 1997;43:135-46.

Rasmussen KLR, Timme A, Suomi SJ. Comparison of physiological measures of Cayo Santiago rhesus monkey females with and between social groups. Primate Rep 1997;47:49-55.

Sameroff AJ, Suomi SJ. Primates and persons: a comparative developmental understanding of social organization. In: Cairns RB, Elder GH, Costello EJ, McGuire A, eds. Developmental science. New York: Plenum Press 1997;97-120.

Sams-Dodd F, Newman JD. Effects of administration regime on the psychomimetic properties of d-amphetamin in the squirrel monkey (Saimiri sciureus). Pharmacol Biochem Behav 1997;56:471-80.

Schmölerich A, Lamb ME, Leyendecker B, Fracasso MP. Mother-infant interactions and attachment security in Euro-American and Central-American immigrant families. Infant Behav Develop 1997;20:167-76.

Schrader L, Hammerschmidt K. Computer-aided analysis of acoustic parameters in animal vocalizations: a multi-parametirc approach. Bioacoustics 1997;7:247-65.

Sternberg, Lamb ME, Dawud-Noursi S. Using multiple informants and cross-cultural research to study the effects of domestic violence on developmental psychopathology: Illustrations from research in Israel. In: Luthar SS, Burack JA, Cicchetti D, Weisz J eds. Developmental psychopathology: perspectives on risk and disorder. New York: Cambridge University Press 1997;417-36.

Suomi SJ. Biological, maternal, and life-style interactions with the psychosocial environment: primate models. In: Hertzman C, Kelly S, Bobak M, eds. Environmental and nonenvironmental determinants of the East-West life expectancy gap in Europe. Amsterdam: Kluver Press 1997;133-42.

Suomi SJ. Nonverbal communication in nonhuman primates: implications for the emergence of culture. In: Molnar P, Segerstrale U, eds. Where nature meets culture: nonverbal communication in social interactions. Mahwah NJ: Erlbaum 1997:131-50.

Suomi SJ. Early determinants of behaviour: evidence from primate studies. Brit Med Bull 1997;53:170-84.

Wang Z, Moody K, Newman JD, Insel TR. Vasopressen and oxytocin immunoreactive neurons and fibers in the forebrain of male and female common marmosets (Calithrix jacchus). Synapse 1997;27:14-25.

Wang Z, Toloczko D, Young LJ, Moody K, Newman JD, Insel TR. Vasopressin in the forebrain of common marmosets (Callithrix jacchus): studies with in situ hybridization, immunocytochemistry and receptor autoradiography. Brain Res 1997;768:147-56.

Westergaard GC, Champoux M, Suomi SJ. Hand preference in infant rhesus macaques (Macaca mulatta). Child Develop 1997;68:387-93.

Westergaard GC, Kuhn HE, Lundquist AL, Suomi SJ. Posture and reaching in tufted capuchins (Cebus apella). Laterality 1997;2:65-74.

Westergaard GC, Lundquist AL, Kuhn HE, Suomi SJ. Ant-gathering with tools by tufted capuchins (Cebus apella). Int J Primatol 1997;18:95-103.

Westergaard GC, Suomi SJ. Capuchin monkey (Cebus apella) grips for the use of stone tools. Am J Physical Anthrop 1997;103:131-5.

Westergaard GC, Suomi SJ. Lateral bias in capuchin monkeys: concordance between parents and offspring. Dev Psychobiol 1997;31:143-7.

LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY

John B. Robbins, M.D., Chief

The LDMI studies the pathogenesis of, and immunity to, bacterial diseases, especially those of infants and children, in order to develop vaccines for their prevention. Since almost all pathogens of healthy individuals are inhabitants for humans only, the LDMI directs most of its efforts at bringing its investigational vaccines to clinical evaluation for safety, immunogenicity and, ultimately, efficacy, as soon as possible. The spirit that guides research of the LDMI is "the proper study of mankind is man."

ENTERIC BACTERIAL INFECTIONS

Vaccine development for enteric pathogens has been hampered because there are hardly any valid animal models for these diseases and because there is controversy about the host immune mechanism(s). The LDMI proposed that a critical level of serum IgG against surface polysaccharides alone is sufficient to confer immunity to bacterial enteric pathogens. We found reports in the literature that serum IgG exudes onto epithelial surfaces, including those of the intestine, and is able to inactivate (lyse) the inoculum of the pathogen, which supports our hypothesis. We developed polysaccharide/protein conjugates because these types of vaccine are effective in inducing protective levels of serum IgG antibodies against polysaccharides in infants and young children.

Conjugate Vaccines Containing the Vi Capsular Polysaccharide of *Salmonella typhi*. The capsular polysaccharide of *Salmonella typhi* (Vi) or the O-specific polysaccharide domain of lipopolysaccharides of nontyphoidal *Salmonella*, *Shigella*, or *Escherichia coli* are both essential virulence factors and protective antigens for these enteric pathogens. These surface polysaccharides "shield" the bacterium from the lytic actions of serum complement alone; antibody is required to induce bacteriolysis.

The Vi capsular polysaccharide of *S. typhi* (poly α(1→4)-D-Gal*p*NAcA, *N*-acetylated at C2 and O-acetylated at O-3), is licensed as a vaccine by the FDA and about 40 countries, and the World Health Organization has published Requirements for this new typhoid vaccine. Similar to other capsular polysaccharides, the limitations of Vi as a vaccine are its age-dependent and T cell-independent immunologic properties.

Following clinical studies in the U.S., Vi was bound to carrier proteins either using a heterobifunctional thiolating linker, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), or the symmetrical bifunctional linker adipic acid dihydrazide (ADH). The carboxyl groups of Vi were derivatized with cystamine (about 1-3%), and this derivative was linked to proteins with SPDP. The carrier, recombinant exoprotein A from *Pseudomonas aeruginosa* (rEPA), was derivatized with ADH and bound to Vi with a water soluble carbodiimide. When injected subcutaneously as a saline solution, the Vi conjugates elicited higher antibody levels than Vi alone and a booster response in guinea pigs and mice. In Vietnam, volunteers, 14-18 years old were injected twice with one of the Vi conjugates or Vi. None of the 165 vaccinees had fever or significant local reactions. The Vi conjugate prepared with ADH elicits higher and longer lasting antibody levels than the other Vi conjugate or Vi alone ($p = 0.01$). Both Vi conjugates are safe and immunogenic in 2- to 5-year-old children. Similar to results obtained in the young adults, the Vi conjugate prepared with ADH elicits significantly higher levels of serum IgG anti-Vi than the conjugate prepared with SPDP. More importantly, this conjugate elicits higher levels of IgG anti-Vi in children than the Vi elicited in adults. Clinical efficacy trials are planned for this year.

Synthetic Typhoid Vaccine. Pectin, a plant polysaccharide, is mostly a homopolymer of (1→4)-α-*D*-Gal*p*A with ~5% neutral sugars and has an average molecular weight ~400 kDa. It differs from Vi in that it lacks an *N*-acetyl at the C2 and O-acetyl at the O-3 positions. Pectin and Vi do not cross-react by immunodiffusion. Treatment of pectin with acetic anhydride results a di-O-acetyl derivative at O-2 and O-3. This derivative is antigenically identical to Vi. Unlike Vi, O-acetylated pectin (OAcPec) is not immunogenic in mice, probably because of its low molecular weight. OAcPec conjugated to tetanus toxoid, following procedures for Vi conjugates (*vide supra*), elicited Vi antibodies in mice and reinjection elicited a booster response. OAcPec is easily prepared, can be measured by standardized colorimetric assays and forms more soluble conjugates than Vi. The use of a plant polysaccharide, pectin, for prevention of a systemic infection caused by *S. typhi* provides a novel approach to improve the preparation and immunogenicity of polysaccharide-based vaccines.

***Salmonella paratyphi* A.** *S. paratyphi* A, second only to *S. typhi* as a cause of enteric fever in Southeast Asia, is a pathogen for, and a habitant of, humans only. Accordingly, *S. paratyphi* is being studied as a model for all nontyphoidal *Salmonella*. The O-specific polysaccharide of *S. paratyphi* A is a polymer of a trisaccharide whose β-L-rhamnose is *O*-acetylated. The lipopolysaccharide (LPS) of *S. paratyphi* A was detoxified by acetic acid hydrolysis, activated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), and bound to tetanus toxoid (TT) with or without a spacer. Solutions of 2.5 μg saccharide, alone or as a conjugate, were injected subcutaneously into young mice and LPS and TT antibodies measured by ELISA. Both conjugates elicit similar levels of serum IgG anti-LPS with bactericidal activity. The conjugate with O-specific polysaccharide activated by CDAP and bound to TT without a spacer elicits the highest level of TT antibodies. Phases 1 and 2 evaluations of *S. paratyphi* A conjugates in Vietnam have been completed. About 200 individuals ranging from young adults (n = 20), 15-17 year-olds (n = 108) and 2-5 year-olds (55) received one or two injections of the *S. paratyphi* A conjugates. None of the vaccinees had fever for two days following the injections and local reactions were mild and of short duration. The conjugate without a spacer elicits higher levels of serum IgG anti-LPS at all ages: all volunteers respond with at last a four-fold rise in anti-LPS with bactericidal activity. A Phase 3 clinical double-blind, randomized, and vaccine-controlled trial of Vi and *S. paratyphi* A conjugates is planned for the beginning of 1998.

***Shigella sonnei*.** *S. sonnei* O-specific polysaccharide bound to *P. aeruginosa* exoprotein A (rEPA) was injected into volunteers from the Israel Defense Force: the dose was 25 μg. None of the 576 volunteers experienced significant local symptoms or fever. In a blinded, randomized, vaccine controlled trial, the *S. sonnei* conjugate demonstrated efficacy (74%, 95% CI 27-100, p = 0.006) against culture-positive infection 77 to 155 days after vaccination. In one company, the *S. sonnei* conjugate showed significant protection (42%, 95% CI 4-81, p = 0.043) against culture-positive shigellosis occurring 1 to 17 days after vaccination. This unexpected finding indicates that conjugates could be used to control epidemics. Vaccinees who developed shigellosis caused by *S. sonnei* had significantly lower serum IgG and IgA IgG anti-LPS compared with those who were vaccinated and did not contract disease. *Shigella* and *E. coli* may be considered as one species, and these data provide evidence that our conjugate vaccines, designed to induce serum IgG anti-LPS, will be effective against enteric bacterial infections (*vide infra*).


John Robbins

Shigellosis is mainly a disease of children and is a major cause of stunted growth throughout the world. Following our demonstration in young adults of safety, immunogenicity, and efficacy of our Shigella conjugates, a clinical trial was conducted in five- to seven-year-old children in the Sheba Medical Center, Israel. 147 children were recruited over a nine-month period and vaccinated on a random basis with either *S. flexneri* type 2a-rEPA, *S. sonnei*-rEPA or hepatitis B (control vaccine). Blood samples were taken before vaccination, six weeks later (immediately before second injection), four weeks after the second immunization and six to eight months after the start of the protocol. No vaccinees had fever equal to or above 38°C or serious local signs, and none had abnormal values for SGOT, SGPT, and alkaline phosphatase. The vaccines elicit a specific and statistically significant rise of serum IgG anti-LPS after each injection. A clinical efficacy trial in one- to three-year-old children awaits the results of our Phase 1 trial of these two Shigella vaccines using new carrier proteins.

***E. coli* O-157 and O-111.** We are studying how to prevent and treat infections caused by *E. coli* O-157, a frequent cause of severe enteritis and the extraintestinal complication of hemolytic uremic syndrome (HUS) especially in children. *E. coli* O157:H7 live in cattle without causing symptoms. There is little information about the host mechanism of immunity because there are no valid animal models for infections caused by *E. coli* O-157 or for the related causes of hemorrhagic colitis and HUS such as *S. dysenteriae* type 1. Our data from the study of *Shigella* suggests that a "critical" level of serum IgG antibodies with bactericidal activity against the O-specific polysaccharide of *E. coli* O-157 may confer immunity to enteritis caused by this pathogen. We prepared O-specific polysaccharide conjugates for *E. coli* O-157 and *E. coli* O-111. Both experimental vaccines have clinically acceptable levels of "endotoxin" and, in mice, both conjugates, when injected with clinically relevant dosages and routes, induce IgG with bactericidal activity for their homologous organisms.

Phase 1 clinical studies showed O-157 conjugates to be safe (no fever or significant local reactions) and to induce significant rises of IgG antibodies with bactericidal activity in all the 75 adult volunteers. About 75% of these adults responded with at least a four-fold rise one week after injection and required only one injection to achieve maximal levels of vaccine-induced antibodies.

Antibiotic treatment does not favorably affect the course of the enteritis and may increase the incidence of HUS, possibly by suddenly increasing the amount of toxin released from the organisms. Conjugates were prepared with the B subunit of Shiga toxin I. A method was developed for the rapid isolation of pure B subunit of Shiga toxin using affinity chromatography based on the blood group P1 antigenic trisaccharide. This trisaccharide, a receptor-binding analog of Shiga-toxins, was synthesized and covalently attached to an agarose-based support. The B subunit of *Shigella* toxin was prepared from the supernatant of a culture of the recombinant nonvirulent *V. cholerae* strain 0395-N1 (prepared by Arthur Donahue-Rolfe of Tufts University School of Veterinary Medicine and Stephen Calderwood of the Massachusetts General Hospital). The resultant conjugates elicited both anti-LPS and neutralizing antibodies (antitoxin). Clinical studies in children are planned as part of our objective to include *E. coli* O-157 and O-111 vaccines in the routine vaccine formulation and to prepare hyperimmune globulin containing IgG anti-LPS and antitoxin as therapeutic agents.

IMPROVING THE IMMUNOGENICITY OF O-SPECIFIC POLYSACCHARIDE PROTEIN CONJUGATES

In conjugate vaccines, the carrier protein also contributes to their immunogenicity. In general, the more immunogenic the carrier, the higher the levels of anti-saccharide antibodies elicited by the conjugate. We have examined a new carrier and a method for increasing the yield of saccharide bound to this protein. *Corynebacterium diphtheriae* CRM9, a genetically-derived non-toxic mutant of C. *diphtheriae*, is being considered as the carrier for the O-specific polysaccharides of *S. flexneri* type 2a and *S. sonnei* for several reasons. First, diphtheria toxoid (formalin-treated diphtheria toxin) and another mutant non-toxic cross-reacting diphtheria toxin protein designated CRM197, are licensed as carriers for *Haemophilus influenzae* type b polysaccharide. Second, clinical experience shows CRM9 to be safe and immunogenic. Third, unpublished clinical data show that formulation of two conjugates using the same carrier protein (*r*EPA) in a syringe may result in a significant reduction in the antibody responses to the saccharide components. Since we wish to have a trivalent formulation for shigellosis (*S. dysenteriae* type 1, *S. flexneri* type 2a and *S. sonnei*), we are studying the use of an unique carrier for each O-specific polysaccharide. CRM9 conjugates induce significantly higher levels of anti-*S. flexneri* type 2a O-specific polysaccharide in mice than do conjugates of this saccharide with *r*EPA. Clinical lots of *S. flexneri* type 2a and *S. sonnei* O-specific polysaccharides have been bound to CRM9 and will be evaluated in adults (Phase 1).


Rachel Schneerson

Another approach has been explored for increasing the immunogenicity of our carrier proteins. The immunogenicity of the saccharide or "hapten" component is related to several properties of conjugates: (1) the higher the density of the saccharide component in the conjugate, the greater the immunogenicity for eliciting antibodies to that component; (2) the higher the molecular weight of the saccharide, the greater the immunogenicity of that component; (3) the more intact the native structure of the carrier protein, the greater the immunogenicity of the saccharide component. We have reached the maximum molecular weight of the O-specific polysaccharide component of our conjugates. Therefore, we have tried to increase the density of the saccharide bound to and the immunogenicity of our carrier protein. The saccharide component is bound to the carrier by 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC), a water-soluble carbodimiide that catalyzes the formation of an amide bond between amino and carboxylic acids. This property of EDC may also result in intramolecular cross-linking, which results in denaturation of the carrier protein. Accordingly, we treated the proteins with low levels of succinic acid anhydride, which converted about eight lysine residues to carboxyls. Addition of carboxyls by succinylation should facilitate the binding of the hydrazide-derivatized O-specific

polysaccharide to the carrier protein. Succinic acid anhydride (dihydro-2,5-furandione) reacts rapidly with ε-amino groups of lysines and α-amino groups of the N-amino acid terminus of proteins in aqueous solutions at neutral pH to form an amide bond with carboxyls. In fact, treatment with succinic acid anhydride has been proposed as a method for both inactivating diphtheria and tetanus toxins and stabilizing the resultant toxoids against aggregation. Succinic acid anhydride is not listed as a mutagen or as a carcinogen. Following successful experiments in mice, five conjugates were synthesized for clinical evaluation:

S. flexneri 2a-*r*EPA$_{succinimyl}$

S. flexneri 2a-CRM9$_{succinimyl}$

S. sonnei-CRM9

S. sonnei-*r*EPA$_{succinimyl}$

S. sonnei-CRM9$_{succinimyl}$

These lots have been approved for Phase 1 testing in adults, and their specifications have been recently submitted to the FDA. Information from these studies will be applicable to all polysaccharide-protein conjugates including those of *E. coli* O-157 and *Vibrio cholerae*.

Synthetic Approach. There are many reasons, based upon immunologic studies, that synthetic saccharides of defined length and homogeneous sites for conjugation would exceed the immunogenicity of the saccharide component prepared from the organism for our conjugate vaccines. Oligosaccharides corresponding to the O-specific polysaccharide of *S. dysenteriae* type 1 have been synthesized and their conformation and binding properties to monoclonal antibodies and to LPS extracted from the bacteria were examined by physicochemical methods. Saccharides containing dimers, trimers, and tetramers of the tetrasaccharide repeating unit, corresponding to octa-, dodeca-, and hexadecasaccharides, have been synthesized. A new heterobifunctional linker containing a free carboxyl group and a masked aldehyde moiety was developed and used for the covalent attachment of the synthetic saccharides to human serum albumin at multiple sites on the protein. An amino group, installed at the terminus of the aglycon of the synthetic saccharides, was acylated with the spacer using carbodiimide activation. Next, the protecting groups of the aldehyde function of the linker were removed by mild acid hydrolysis. Finally, the saccharide-spacer construct was bound to the protein by reductive amination. Conjugates were prepared with saccharides of varying chain lengths and density. The immunogenicity of these conjugates is under investigation.

Heparin and Sulfated Oligoxylans. Sulfated saccharides, especially heparin and sulfated xylans, interfere with the ability of HIV-1 to infect CD4 lymphocytes. Xylans that have been sulfated represent a heterogeneous mixture of polysaccharides. Techniques has been developed to isolate a sulfated xylan that does not have anticoagulant properties but retains the ability to inhibit HIV-1 to infect CD4 lymphocytes. We are planning clinical testing of this sulfated polysaccharide.

PERTUSSIS

In addition to the finding that the NICHD monovalent pertussis toxoid is highly safe and confers immunity to pertussis, we have, in collaboration with Birger Trollfors, John Taranger and Teresa Lagergård in Göteborg, Sweden, made two important observations. First, there is an artifact created when serologic tests for pertussis use components in the vaccine. The criteria for diagnosis of the World Health Organization requires at least 21 days of paroxysmal coughing and isolation of *Bordetella pertussis* or a significant rise of serum IgG anti-pertussis toxin or anti-filamentous hemagglutinin. We showed that vaccination with pertussis toxoid reduces the yield of *B. pertussis* and, therefore, greater reliance is placed on the serologic diagnosis of pertussis. About 90% of pertussis patients, both vaccinated with the pertussis toxoid or controls (recipients of diphtheria and tetanus toxoids) respond with a significant rise in anti-FHA. In contrast, only about 25% of vaccinees respond with a rise of anti-pertussis toxin whereas about 90% of controls respond to this antigen. Accordingly, the serologic assays artificially lower the number of cases among recipients of multivalent vaccines that contain pertussis toxoid and FHA. Second, we have demonstrated "herd" immunity (protection of non-vaccinated individuals) among family members of vaccinees. This last finding, along with the demonstration that widespread vaccination of the remainder of children five years of age and older in Göteborg has resulted in a statistically significant decline in the number of cases in that city, provide further evidence that pertussis toxoid alone is

both necessary and sufficient for the new pertussis vaccine and that this safe product is suitable for addition to diphtheria and tetanus toxoids for routine vaccination of adults in order to eliminate that reservoir of *B. pertussis* remaining in this age group.

LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY

Chief
John B. Robbins, M.D.

Principal Investigator
Rachel Schneerson, M.D.

Staff Scientists
Arthur B. Karpas, Ph.D.
Shousun C. Szu, Ph.D.
Vince Pozsgay, Ph.D.

Post-Doctoral Fellow
Zuzana Kossaczka, Ph.D.

Senior Technical Specialist
Dolores Bryla

Senior Fellows
Slavomir Bystricky, Ph.D.
Jean Mukherjee, Ph.D.

Pre-Doctoral Fellow
Abraham Lin

Guest Researchers
Zhongdong Dai, M.D.
Chiayung Chu, M.D.
Jianping Li, M.D.
C. Goran Ekborg, Ph.D.
Audrey Stone, Ph.D.

ANNUAL BIBLIOGRAPHY

Bystricky S, Pavliak V, Szu SC. Characterization of colominic acid by circular dichroism and viscosity analysis. Biophys Chem 1997;63:147-52.

Cohen D, Ashkenazi S, Green MS, Gdalevich M, Robin G, Slepon R, Yavzori M, Orr N, Block C, Ashkenazi I, Shemer J Taylor DN, Hale TL, Sadoff JC, Pavliakova D, Schneerson R, Robbins JB. Double-blind vaccine-controlled randomised efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. Lancet 1997;349:155-9.

Cohen D, Ashkenazi S, Green M, Lerman Y, Slepon R, Robin G, Orr N, Taylor DN, Sadoff JC, Chu C, Shiloach J, Schneerson R, Robbins JB. Safety and immunogenicity of investigational *Shigella* conjugate vaccines in Israeli volunteers. Infect Immun 1996;64:4074-7.

Cohen D, Ashkenazi S, Green M, Gdalevich M, Yavzori M, Orr N, Robin G, Slepon R, Lerman Y, Block C, Ashkenazi I, Taylor D, Hale L, Sadoff J, Schneerson R, Robbins J, Wiener M, Shemer J. Clinical trials of Shigella vaccines in Israel. Adv Exp Med Biol 1996;397:159-67

Coxon B, Sari N, Mulard LA, Kovac P, Pozsgay V, Glaudemans CPJ. Investigation by NMR spectroscopy and molecular mechanics of the conformations of some modified disaccharide antigens for *Shigella dysenteriae* type 1. J Carbohydr Chem 1997;16:927-46.

Dubois EP, Robbins JB, Pozsgay V. Chemical approaches to bacterial vaccines. Synthesis of mycobacterial oligosaccharide-protein conjugates for use as serodiagnostics and immunogens. Bioorg Med Chem Lett 1996;6:1387-92.

Gu X-X, Sun J, Jin S, Barenkamp SJ, Lim DJ, Robbins JB, Battey J. Detoxified lipooligosaccharide from nontypeable *Haemophilus influenzae* conjugated to proteins confers protection against otitis media in chinchillas. Infect Immun 1997;65:4488-93.

Gu X-X, Tsai CM, Ueyama T, Barenkamp SJ, Robbins JB, Lim DJ. Synthesis, characterization, and immunologic properties of detoxified lipooligosaccharide from nontypeable *Haemophilus influenzae* conjugated to proteins. Infect Immun 1996;64:4047-53.

Hardy BJ, Bystricky S, Kovac P, Widmalm G. Conformational analysis and molecular dynamics simulation of a α-(1→2) and α-(1→3) linked rhamnose oligosaccharides: reconciliation with optical rotation and NMR experiments. Biopolymers 1997;41:83-96.

Kiang J, Szu SC, Wang LX, Tang M, Lee YC. Determination of 2-keto-3-deoxyoctulosonic acid (KDO) with high-performance anion-exchange chromatography (HPAEC): survey of stability of KDO and optimal hydrolytic conditions. Anal Biochem 1997;245:97-101.

Klugman KP, Koornhof HJ, Robbins JB, LeCam NN. Immunogenicity, efficacy and serologic correlate of protection of *Salmonella typhi* Vi capsular polysaccharide vaccine three years after immunization. Vaccine 1996;14:435-8.

Kossaczka Z, Bystricky S, Bryla DA, Shiloach J, Robbins JB, Szu SC. Synthesis and immmunological properties of Vi and di-O-acetyl pectin conjugates with adipic acid dihydrazide as the linker. Infect Immun 1997;65:2088-93.

Medgyes A, Farkas E, Liptak A, Pozsgay V. Synthesis of the monosaccharide units of the O-specific polysaccharide of *Shigella sonnei*. Tetrahedron 1997;53:4159-78.

Miller CE, Glaudemans CP, Pozsgay V, Kovac P, Huppi K, Schneerson R, Karpas A. Of four anti-*Shigella dysenteriae* type 1 O-polysaccharide murine antibodies, three employ V-genes that are widely divergent from those of the fourth. Mol Immunol 1996;33:1217-22.

Pozsgay V, Dubois EP, Lotter H, Neszmelyi A. Synthesis and x-ray crystallographic study of cyclobis-(1→2)-α-D-glucopyranosyl peracetate. Carbohydr Res 1997;303:165-73.

Pozsgay V, Dubois EP, Pannell L. Synthesis of kojidextrins and their protein conjugates. Incidence of steric mismatch in oligosaccharide synthesis. J Org Chem 1997;62:2832-46.

Robbins JB, Schneerson R, Anderson P, Smith DH. Prevention of systemic infections, especially meningitis, caused by *Haemophilus influenzae* type b: impact on public health and implications for other polysaccharide-based vaccines. JAMA 1996;276:1181-5.

Robbins JB, Schneerson R, Szu SC. New ideas about vaccine-induced immunity to otitis media. In: Recent Advances in Otitis Media. BC Decker, Inc.: Hanover, 1996;8-9.

Robbins JB, Schneerson R, Szu SC. O-specific polysaccharide-protein conjugate for prevention of enteric bacterial diseases. In: Levine MM, Woodrow GC, Kaper JB, Cobon GS, eds. New generation vaccines, 2nd Edition. Marcel Dekker, Inc.: New York, 1997;803-15.

Robbins JB, Towne DW, Gotschlich EC, Schneerson R. "Love's labours lost": failure to implement mass vaccination against group A meningococcal meningitis in sub-Saharan Africa. Lancet 1997;350:880-2.

Schneerson R. Immunogenicity and safety of mixed DTP/IPV/Hib vaccines [letter; comment]. Lancet 1997;349:881-2.

Schneerson R, Robbins JB. Ukraine's diphtheria campaign [letter]. Lancet 1996;348:1245.

Schneerson R, Robbins JB, Taranger J, Lagergard T, Trollfors B. A toxoid vaccine for pertussis as well as diphtheria? Lessons to be relearned. Lancet 1996;348:1289-92.

Schneerson R, Robbins JB, Taranger J, Lagergard T, Trollfors B. Examination of similarities between diphtheria and pertussis and their toxoids provide insight into vaccine-induced protection from *Bordetella pertussis* infection. Dev Biol Stand 1997;89:321-6.

Schneerson R, Robbins JB, Taranger J, Trollfors B, Lagergard T. Toxoid vaccine for pertussis. Lancet 1997;349:137.

Sigurdardottir ST, Vidarsson G, Gudnason T, Kjartansson S, Kristinsson KG, Jonsson S, Valdimarsson H, Schiffman G, Schneerson R, Jonsdottir I. Immune responses of infants vaccinated with serotype 6B pneumococcal polysaccharide conjugated with tetanus toxoid. Pediatr Infect Dis J 1997;16:667-74.

Taranger J, Trollfors B, Lagergard T, Lind L, Sundh V, Zackrisson G, Bryla DA, Robbins JB. Unchanged efficacy of a pertussis toxoid vaccine throughout the two years after the third vaccination of infants. Pediatr Infect Dis J 1997;16:180-4.

Trollfors B, Taranger J, Lagergard T, Lind L, Sundh V, Zackrisson G, Bryla DA, Robbins JB. Efficacy of a monocomponent pertussis toxoid vaccine after household exposure to pertussis. J Pediatr 1997;130:532-6.

LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

Phillip G. Nelson, M.D., Ph.D., Chief

Members of the Laboratory of Developmental Neurobiology (LDN) continue to play a leadership role in developmental neuroscience, as exemplified by the numerous symposia and sessions at national and international meetings that they have organized and chaired. LDN staff have provided the first evidence for a sorting signal-receptor mediated mechanism for sorting prohormones to the regulated secretory pathway. They have identified the peptide sorting motif and have shown that the receptor for this motif is membrane-bound carboxypeptidase E. They have continued the analysis of a novel class of prohormone converting aspartic proteases. The early steps in the process of activity-dependent synapse elimination in the neuromuscular synapse have been identified *in vitro*. Muscle activation produces thrombin, a serine protease that activates the thrombin receptor, which in turn up-regulates protein kinase C. The targets for PKC action are presently unknown but may include the acetyl choline receptor. LDN staff have shown that regulation of the key enzyme in melatonin synthesis, serotonin N-acetyltransferase (AANAT), always involves an increase in enzyme protein and usually an increase in AANAT RNA (150 fold in the rodent). In sheep, no such change in mRNA levels occurs. Additional AANAT genes have been identified in fish. Another LDN group has shown that sialogangliosides are a functional component of the tetanus toxin receptor. One clostridial neurotoxin, the C1 serotype, has a uniquely degenerative effect on mature neurons, perhaps due to the closè relationship of its target protein, syntaxin, to calcium channels. Members of the LDN have shown that the developmental activation of the genes for the NR2B subunit of the NMDA receptor in the cerebellum requires two converging signals: neuregulin (or ARIA) and activation of the NMDA receptor. They have identified regulatory sequences on the fast and slow muscle troponin genes that mediate the response to activity patterns in the muscle that determine fast or slow fiber types. LDN workers have further shown that progression from pluripotent neuroepithelial cells to neurons and glia is accompanied by the appearance of two proteins characterizing neurons and glia, which bind to a homodimer of 33 kDa found in the precursor cells. Different DNA bending is produced by the three complexes. Another project concerns the modulation of neuron and glial gene expression by specific patterns of electrical activity in nerves. In particular, cell adhesion molecules such as NCAM, L-1 and N cadherin have been examined, all demonstrating different sensitivity to action potential patterns. Myelination is also activity-dependent. Some of the cell-biologic kinetics that might explain pattern sensitivity are under investigation. LDN staff have pioneered the area of neurotrophic factor regulation of synaptic transmission. They have shown that BDNF regulates hippocampal long-term potentiation by a presynaptic mechanism. Expression of a neurotrophic molecule NT-3 is regulated by activity in skeletal muscle and may serve as a retrograde messenger for activity-dependent synaptic strengthening at the developing neuromuscular junction.

NEUROTROPHIC FACTORS

The Section on Developmental and Molecular Pharmacology, led by ***Douglas Brenneman***, continues to focus on the neurotrophic and growth-regulating actions of vasoactive intestinal peptide (VIP). Previous studies by this group have shown that many of the developmental events regulated by VIP are mediated through substances released by this neuropeptide. Much of the effort has been on identifying these intermediary substances and studying their mechanism of action. This research has resulted in the discovery a new class of neuroprotective molecules: activity dependent neurotrophic factors (ADNFs). Importantly, peptide fragments of the ADNFs are biologically active and have become lead compounds for drug development to treat neurodegenerative disease. We have identified a nine-amino-acid peptide fragment of our previously isolated activity dependent neurotrophic factor (ADNF), which works through receptor-mediated endocytosis and which has neuroprotective effects when administered intranasally. Because the biological action of the ADNFs are dependent on and interactive with the electrical activity of the target nerve cells, this line of research is consistent with and contributes to the overall focus of the Laboratory of Developmental Neurobiology on the regulatory role of synaptic activity in nervous system development. Given the remarkable growth-stimulating action of VIP, another significant aspect of this section's effort is on the physiological role of VIP during pregnancy. Maternal-fetal interactions have been shown to be mediated in part by VIP and early pregnancy factor (EPF).

Pharmacology of Femtomolar-Acting Neurotrophic Peptides. With the discovery of a neuroprotective, nine-amino-acid peptide derived from ADNF I, mechanistic studies on this unusually potent peptide (ADNF-9) were initiated. In collaboration with Elaine Neale (LDN), we examined the effects of inhibitors of receptor-mediated endocytosis (RME) on cultures treated with ADNF-9. Initial studies were done with bafilomycin A1 (BFA1), a specific inhibitor of vacuolar H^+- ATPase and a drug that has been used to study the role of RME. Short term co-treatment with BFA1 was shown to prevent the survival-promoting action of ADNF-9. These studies supported the hypothesis that ADNF-9 works through an RME mechanism. Other work compared the action of ADNF-9 in cultures consisting of neurons and abundant astroglia with cultures composed primarily of neurons. These studies revealed several important features of ADNF-9 pharmacology. Apparently, this peptide can act directly on neurons to increase their survival. However, the potency of the response is 10,000 time less in the absence of astroglia. These studies strongly imply that ADNF-9 interacts with both neurons and astroglia to enhance neuronal survival at femtomolar concentrations. Kinetic experiments revealed another important facet to ADNF-9 action: a very short term exposure (less than two hours) to this peptide results in neuroprotection for cerebral cortical neurons that persists for four to five days after treatment. In addition, Illana Gozes, a collaborator at Tel Aviv University, has shown that this peptide has neuroprotective action when given intranasally to animals that are undergoing chemically induced neurodegeneration. These findings have obvious implications for the development of this peptide as a therapeutic agent and directly addresses two pharmacokinetic problems: duration of action and drug delivery to the brain. Although further work is required to optimize the composition of the ADNF peptides, this international team has developed a promising and novel strategy for intervention in neurodegenerative disease of the central nervous system.


Douglas Brenneman

VIP: Role in Maternal-Fetal Interactions. Our previous work revealed that VIP can orchestrate the growth of an entire embryo during the early postimplantation period of development. These observations, carried out in whole embryo cultures, indicated that VIP could have a hormonal role of growth regulation during a very specific period of gestation. Further studies showed that administration of a VIP antagonist during the time of neural tube closure in the rat produces microcephaly, while sparing body growth. Measurements of mRNA for VIP during development indicate that mouse embryos do not synthesize VIP until relatively late in pregnancy, although abundant VIP immunoreactivity is present. If VIP does indeed have growth-regulating properties, an important question remains the source of VIP during this critical period, as is the changing in expression of VIP from maternal tissues to embryo. We found that at E8 VIP mRNA is abundant in the trophoblast, but that the peptide message is undetectable in the embryo. Trophoblastic VIP mRNA decreases until E10, after which it cannot be detected. Both RT-PCR and *in situ* hybridization revealed VIP message in the decidua of the uterus. Dense VIP binding sites, as demonstrated with VIP autoradiography, are evident in the trophoblast and the decidua as early as E6. Interestingly, these VIP binding sites are also transient, becoming undetectable after E10. Taken together, these data indicate that both VIP and its binding sites are expressed in a transient manner during a period in development in which VIP has a dramatic influence on embryonic growth. These data support the hypothesis that extraembryonic sources of VIP may be an important regulator of embryonic growth.

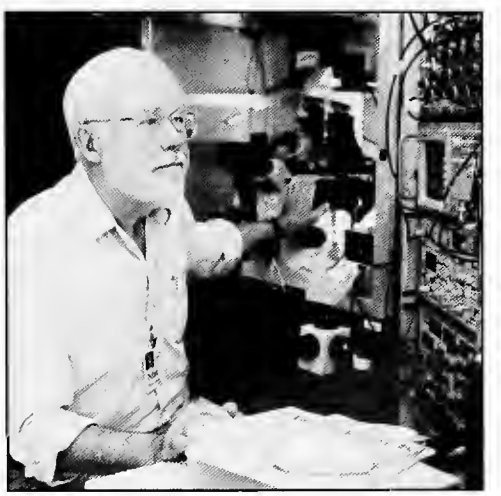
Early Pregnancy Factor. Previous studies with ADNF have shown that this protein has both structural homologies to heat shock protein 60 and that it can increase the growth of postimplantation embryos. In related studies, we have examined another extracellular heat shock protein, early pregnancy factor (EPF). Treatment of whole embryo cultures of E9 mice with EPF results in significant increases in somite number. Measurements of DNA and protein showed that there is a proportionately greater increase in brain growth compared with body growth in EPF-treated embryos. Based on our previous work with ADNF, a search was conducted for active peptides derived from EPF. An active 20 amino acid peptide derived from EPF was discovered. Studies on the neuroprotective and growth-promoting actions of this peptide are under way. Taken together, these data indicate that two secreted heat-shock-protein-like substances have growth-regulating properties. In addition to effects on embryonic growth, the expression of EPF in the brain of adult mice was examined. We detected EPF message throughout the brains of adult male and female mice by *in situ* hybridization. Especially intense labeling was

observed in the thalamic nuclei and cortex during pregnancy. EPF mRNA is also present in the embryonic neuroepithelium and in the surrounding amniotic and trophoblastic membrane from E6 to E12. This protein apparently has functions in both the reproductive and the central nervous system during pregnancy.

DEVELOPMENT OF SYNAPTIC CIRCUITS

Activity-Dependent Development of Synaptic Circuits. ***Phillip Nelson*** and his colleagues of the Section on Neurobiology have elucidated the initial steps in the process of activity-dependent synapse elimination that occurs at the neuromuscular junction (NMJ), using a three-compartment tissue culture system. Two populations of cholinergic neurons innervate skeletal muscle in this system, and we show that unilateral activation of one population of the innervating neurons produces substantial reduction in the innervation of doubly innervated muscle cells and that this elimination is relatively selective for the inactive input. This is in accord with the behavior of a Hebb synapse. Activation of muscle results in the elaboration of thrombin, a serine protease, and exogenous thrombin induces synapse loss. A specific thrombin inhibitor, hirudin, blocks activity-induced loss. Thrombin may act through a thrombin receptor, and a thrombin receptor-activating peptide (TRAP) can induce synapse loss. We show that this action is mediated by protein kinase C (PKC), since blockers of PKC, including the highly specific blocker calphostin C, prevents both TRAP and activity-induced synapse loss. A specific protein kinase A blocker (H-89) has no blocking effect. Thus, muscle activation produces an increase in thrombin activity which, via a PKC-mediated mechanism, affects synapse efficacy. The targets for PKC action probably include the acetylcholine receptor.

We have investigated the mRNA levels of prothrombin expression in the developing mouse leg muscle, because of the possible relevance of thrombin action in synapse elimination at the neuromuscular junction *in vivo*. The mRNA levels of thrombin, of the thrombin receptor (ThR), and of the endogenous thrombin inhibitor protease nexin 1 (PN-1) have been measured in an attempt to establish the interrelations of these molecules, because of their possible involvement in synapse elimination at the neuromuscular junction (NMJ). In addition, we measure the mRNA levels of the three genes in muscle after denervation to study the influence of innervation on the expression of these molecules. We found that the levels of prothrombin and thrombin receptor are high at birth, but drastically fall to a very low level at postnatal day 20, which coincides with the period when stable synapses have been established at the rodent NMJ. The level of PN-1 remains fairly constant during muscle development except for a decrease around postnatal days 10 and 15, which coincides with the period during which most synapse elimination occurs in the rodent NMJ. Denervation results in a reversal of the developmental regulation of the genes. The mRNA levels of prothrombin and its receptor significantly increase while the expression of PN-1 mRNA decreases. The concurrent regulation of prothrombin and ThR suggests that thrombin-mediated cellular activities in muscle are affected by the activation of ThR, as shown in many other thrombin-mediated cellular effects. An elevated level of thrombin or of thrombin-like activity might result from the decrease in inhibitory activity of PN-1 during the period of peak synapse reduction during muscle development.



Phillip Nelson

Activity-Independent Establishment of Synaptic Circuits. The neuronal projection between the eye and its main target in the CNS, the optic tecta (chick) or superior colliculus (mammals) results in the precise two-dimensional, contralateral projection. We have focussed on the behavior of retinal ganglion cell (RGC) fibers following contact with target (optic tecta or superior colliculus) cells.

RGC growth cones forced to encounter dissociated target cells in a coculture environment exhibit one of three characteristic behaviors: they collapse, stop, or continue unimpeded outgrowth. We previously showed that the type of growth cone behavior exhibited depends on whether the RGC growth cone contacts a neuron or glial cell, and whether the cell was isolated from a chick or mouse. Molecules which can mediate the collapse response were identified by other laboratories in the chick optic tectum. During this past year, we localized the RNA for these molecules to the neuronal and glial populations of both the chick optic tectum and mouse

superior colliculus. One of the molecules, ephrin-A2, is distributed on neurons in both systems and can account for much of the observed repulsive growth cone behavior. The second molecule, ephrin-A5, is distributed on both neurons and glia in the chick but is present in much lower amounts on mouse neurons. These expression patterns, together with the previous growth cone behavior studies, suggested a model of the sequential cell-cell interactions that are critical to the development of the retino-optic map that forms across both the chick optic tecta and rodent superior colliculus. Interestingly, known differences in the development of avian retino-tectal and rodent retino-collicular maps can be accounted for by the same minimal set of cellular interactions.

The elimination of the ipsilateral retino-tectal projection is nearly complete in the chick embryo, and therefore, factors which might prevent or prolong the elimination of projections in the CNS can be readily studied. We examined two types of reagents, protease inhibitors and trophic factors, for their ability to prevent elimination of the ipsilateral retino-tectal projection in chick. Our laboratory has previously shown that serine protease inhibitors prevent synaptic elimination of the neuromuscular junction. These reagents can also prolong the retino-tectal connection; however, the projection is completely lost in developmentally advanced embryos. Trophic factors have been implicated in a number of systems in preventing cell death and, at the neuromuscular junction, in limiting the degree of synaptic elimination. BDNF applied to the eye is able to completely prevent loss of the retinal projection to the ipsilateral tecta. This suggests that trophic factors can prevent the natural loss of CNS projections but also that the action of trophic factors at the cell body may be a strong determinant of the maintenance of the projection in distal targets.

BIOSYNTHESIS, PROCESSING, AND SECRETION OF NEUROPEPTIDES AND PITUITARY PEPTIDE HORMONES

The Section on Cellular Neurobiology, led by ***Peng Loh***, studies the intracellular trafficking, enzymology and regulation of biosynthesis of neuropeptides and prohormones, with specific focus on the ACTH/endorphin family of peptides. These peptides play a role in intercellular communication and neural development. Peptides belonging to the ACTH/endorphin family are cleaved from a common prohormone, pro-opiomelanocortin (POMC). Like other prohormones, POMC is synthesized at the rough endoplasmic reticulum, transported to the Golgi apparatus, and sorted at the trans-Golgi network (TGN) into secretory granules of the regulated secretory pathway, where it undergoes proteolytic processing and subsequent regulated secretion of the products. A highly specific set of proteases are involved in cleaving this prohormone, in a tissue-specific manner, to yield different peptide products in brain, intermediate lobe, and anterior lobe of the pituitary.

Mechanism of Sorting Prohormones to the Regulated Secretory Pathway. The Section continues to investigate the molecular signals and mechanism(s) involved in the sorting of prohormones and pro-neuropeptides to the secretory granules of the regulated secretory pathway, using POMC as a model system. A sorting signal motif consisting of a 13 amino acid (N-POMC$_{8\text{-}20}$) amphipathic loop stabilized by one disulfide bridge (Cys-8/Cys-20), which is both sufficient and necessary for targeting POMC to the regulated secretory pathway, has been identified. Molecular modeling studies done in collaboration with C. Snell (Novartis Inst., London) show that the loop contains two pairs of acidic/hydrophobic residues, Asp-10/ Leu-11 and Glu-14/ Leu-18, exposed on the surface, that may be involved in ionic and hydrophobic interactions with a sorting receptor. Substitution of these four residues with Ala-10, Ser-11, Ala-14, and Ser-18 eliminates the targeting of this POMC mutant to the regulated secretory pathway and binding to secretory granule membranes, underscoring the physiological importance of these four residues in sorting POMC at the TGN. A similar sorting signal motif was also identified in the N-terminal of pro-enkephalin, within residues 1-32, by molecular modeling. This motif consists of two amphipathic loops stabilized by two disulfide bridges, Cys-2/Cys-24 and Cys-6/Cys-28, with two pairs of conserved acidic/hydrophobic residues (Asp-18/Ile-19, and Glu-29/Leu-32) exposed on the surface. When fused to chloramphenicol acetyltransferase and transfected into neuroendocrine cells (neuro2a), N-proenkephalin 1-32 is able to direct this bacterial protein to the regulated secretory pathway, confirming the predictions of the model. Disruption of the Cys-2/Cys-24 disulfide bridge however caused misrouting of this fusion protein to the constitutive pathway, indicating the conformational dependence of this motif.

Binding and cross-linking studies of the N-POMC sorting signal to secretory granule membranes identified a specific sorting receptor for this signal. Subsequent purification and N-terminal amino acid sequencing of the sorting receptor protein identified it as membrane bound carboxypeptidase E, (CPE). Similar studies showed that proenkephalin and proinsulin also bind specifically to this receptor, but that constitutively secreted proteins do not bind. The binding kinetics (IC_{50}) of all three prohormones POMC, proenkephalin, and proinsulin are similar, and Scatchard analysis revealed binding characteristic of a low affinity, single binding site type of receptor.

Down-regulation of CPE by antisense RNA in Neuro2a cells results in the misrouting of these three prohormones to the constitutive pathway, indicating that CPE is a common sorting receptor *in vivo*. A mutant mouse, Cpe^{fat}/Cpe^{fat}, lacking CPE due to a mutation in the CPE gene, exhibits constitutive secretion of POMC from the pituitary, further supporting the hypothesis that CPE acts as a sorting receptor for the regulated secretory pathway *in vivo*. These studies provide the first evidence for a sorting-signal-receptor-mediated mechanism for sorting prohormones to the regulated secretory pathway. Uniquely, a membrane-bound exopeptidase, CPE, serves as the sorting receptor, which subsequently plays a second role, after packaging into the secretory granule, as a processing enzyme for trimming off the C-terminally extended basic residues from the cleaved hormones. Thus, a genetic defect in the CPE gene, as in the Cpe^{fat}/Cpe^{fat} can lead to multiple endocrinological problems, such as hyperproinsulinemia, obesity, and infertility, due to intracellular misrouting of prohormones.

Characterization of Novel Pro-Hormone Converting Aspartic Proteases. Our group has continued to study the proteolytic enzymes involved in the conversion of prohormones to active hormones with a specific focus on a novel class of prohormone converting aspartic proteases. These enzymes have unique specificity for monobasic and paired basic residues of prohormones, and they include yeast aspartic protease 3 (YAP3p/Yapsin1) and mammalian pro-opiomelanocortin converting enzyme (PCE). We showed that Yapsin1 and PCE are structurally related, based on immunological cross-reactivity of antiserum against Yapsin1 with PCE. Immunocytochemistry, in combination with *in situ* hybridization, revealed co-localization of yapsin1-like immunoreactivity with CCK mRNA in hippocampal and cortex neurons, vasopressin mRNA in supraoptic neurons, and POMC in pituitary corticotrophs, indicating a role of Yapsins in prohormone processing in the CNS. We have studied Yapsin1 extensively as a model enzyme of the Yapsin family. Biosynthesis studies of Yapsin1 showed that it is synthesized as an inactive pro-enzyme, and that it is activated by the removal of the pro-region. Analysis of the activation process *in vitro*, using recombinant yapsin1, showed that at an acidic pH the pro-region is first cleaved internally, followed by final removal of the remaining pro-region by cleavage at Arg-67. Both cleavages are autocatalytic and intramolecular; pepsin cleaves in a similar manner. In yeast, Yapsin1 is further cleaved to yield α and β subunits, which are held together by a disulfide bridge, forming a heterodimer. Surprisingly, pulse-chase studies using wild-type and the sec 18 yeast mutant cells showed that pro-Yapsin 1 is first cleaved into the α and β subunits in the endoplasmic reticulum, followed by removal of the pro-region in the Golgi. The cleavage of Yapsin 1 into the α and β subunits does not occur at a monobasic or at a paired basic residue and is not autocatalytic, accounting for the fact that this cleavage cannot be detected *in vitro* using purified recombinant yapsin1.

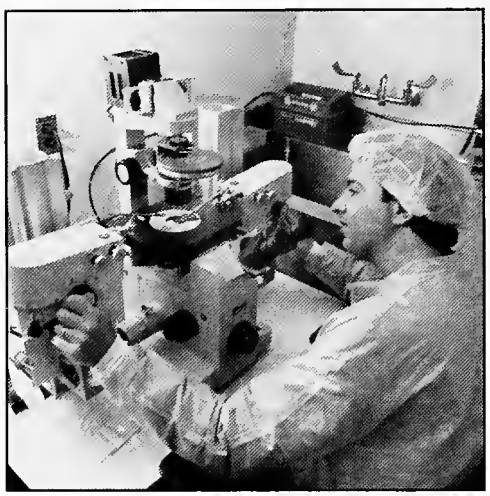
Peng Loh

An extensive study of the specificity of Yapsin1 for basic residues was undertaken. In collaboration with Tom Blundell's group (Cambridge University), a computer model of Yapsin1 was generated, predicting the various amino acids that form the active site pockets of the enzyme. The model shows that the S1 pocket of Yapsin1 is unique and that it differs from all the other aspartic proteases that have specificity for hydrophobic residues in having a more open pocket, facilitating the accommodation of a large amino acid such as a basic residue. Moreover, the S1 pocket contains two acidic residues, which renders it highly electronegative, favoring interaction with basic residues in the P1 position and explaining the basic residue specificity of Yapsin1. The model further predicts that the S6, S2′, S3′, and S6′ active site pockets are also electronegative and that basic residues binding to these pockets would facilitate the efficiency of cleavage. This prediction was borne out by experimental data showing that $CCK_{13\text{-}33}$ analog substrates containing Arg residues at the P6, P2′, P3′ and P6′ positions are cleaved with high catalytic efficiency (k_{cat}/K_m) and that the effects of the substitutions are additive. Arg has the strongest effect in the P2′ position, forcing Yapsin1 to cleave at a non-basic residue in the P1 position, although not efficiently, consistent with the model predicting that the S2′ pocket has three negative charges that could interact with a P2′ Arg. These findings provide major advances in the understanding of the molecular basis of the unique specificity of this class of aspartic proteases.

MOLECULAR MECHANISMS REGULATING NEURAL AND NEUROMUSCULAR PLASTICITY

Nerve-elicited electrical activity is important for the formation of neural circuits during development and maturation of synaptic connections. These activity-dependent processes require the coupling of synaptic signals to selective changes in gene expression. The Unit on Molecular Neurobiology, under the direction of ***Andres Buonanno***, has used cerebellar granule cells and skeletal muscle as model systems to identify factors that mediate activity-transcription coupling.

Electrical Activity and Neural Factors Differentially Regulate NMDA Receptor Gene Expression. The electrophysiological properties of NMDA receptors (NR) are modified by a subunit switch that occurs when granule cells are innervated by glutamatergic mossy fibers, suggesting that either activity or neurally-derived factors, or both, regulates the repression of NR2B expression and activation of the NR2C subunit gene. Using transgenic mice, we found that repression of the NR2B gene is conferred by 1.8 kb of 5'-flanking sequence. Regulatory elements were delineated further in transfected cultures of dissociated granule neurons; these cells develop functional synapses between days 4 and 10 in culture and repress NR2B expression in response to activity. A minimal NR2B promoter construct (-135/+15) that confers neural-specificity and activity-dependent repression was identified. This element is being used to identify transcription factors mediating the activity-dependent repression of the NR2B gene. In contrast to the NR2B subunit gene, NR2C expression increases after granule cell innervation. We found that activation of the NR2C gene requires two converging signals: neuregulin (Nrg) and activity through NMDA receptors. Nrg (also known as ARIA) is a neural factor that accumulates at the glutamatergic mossy fiber/granule cell synapse. We found that Nrg stimulates NR2C expression more than 1,000-fold in cerebellar slice cultures. Addition of the activity inhibitors TTX (sodium channel blocker) or AP-5 (NR blocker) to the slices abolishes the Nrg-dependent stimulation of NR2C; DNQX (an AMPA receptor blocker) has no effect. Consistent with these findings, we found that neuregulin receptors (erbB receptor tyrosine kinases) are expressed by granule cells prior to the NR2B/NR2C subunit switch. These results demonstrate that similar mechanisms and factors (i.e., Nrg) are used to regulate receptor composition in muscle and the CNS during synaptogenesis. Based on our findings, we have proposed a novel mechanism that may be of general importance for selectively regulating neural plasticity during development, i.e., the need for converging signals elicited by molecules that are released from presynaptic neurons to specifically regulate gene expression in the post-synaptic cell.



Andres Buonanno

Distinct Patterns of Electrical Activity Differentially Regulate Muscle Gene Expression. To understand how specific activity patterns regulate gene expression, we have studied how distinct depolarization patterns elicited by motoneurons differentially regulate transcription of either slow-or fast-contractile protein genes in skeletal muscle. Our studies on the muscle troponin I slow (TnIs) and fast (TnIf) genes, which are differentially stimulated by distinct depolarization frequencies (10 vs. 100 Hz, respectively), have focused on the identification of *cis*- and *trans*-acting factors that mediate the specific responses to activity patterns. We have identified a 128 bp "slow" upstream regulatory element (SURE) and a 144 bp "fast" intronic regulatory element (FIRE) that direct either slow- or fast-muscle-specific transcription in transgenic mice. Interestingly, the TnI SURE and FIRE have four common *cis*-acting elements: an A/T-rich sequence (which binds to MEF2), an E box (which binds to MyoD-related factors), a CACC box, and a novel motif (GCAGGCA) that we named the CAGG box. Electrophoretic mobility shift assays with muscle nuclear extracts demonstrate specific binding to these motifs. Functional studies performed in cultured myocytes and transgenic mice demonstrate that interaction of multiple protein-DNA complexes are necessary for enhancer function. Experiments are in progress to identify which of these elements and corresponding transcription factors mediate the frequency-specific response of the TnI genes, and which also participate in frequency-dependent regulation of neural genes.

STRUCTURE/FUNCTION IN NEURONAL CELL CULTURES: STUDIES USING THE CLOSTRIDIAL NEUROTOXINS

The Section on Cell Biology, under the direction of ***Elaine Neale***, has continued to study similarities and differences among the clostridial neurotoxins. These are toxins that cause clinical tetanus and botulinum poisoning by blocking specific neuronal functions. Each of the toxins blocks the release of neurotransmitters, most likely as a consequence of the proteolysis of specific proteins required for the docking and fusion of synaptic vesicles with the presynaptic membrane. These toxins are of interest to neuroscientists and cell biologists because they can be used as tools to modulate synaptic activity and to study membrane movements within cells. Importantly, several of the botulinum toxins are used clinically to relieve the muscle spasms that characterize a variety of disorders.

Gangliosides as Functional Tetanus Toxin Receptors. We have continued studies on the role of gangliosides in mediating the action of tetanus toxin. Each of the clostridial neurotoxins is thought to bind to a specific membrane receptor as a first step in toxin entry into the nerve terminal. Polysialogangliosides on the neuronal surface membrane fix tetanus toxin, although it is not clear that a ganglioside/toxin interaction is required for toxin internalization. We maintained spinal cord cell cultures in the presence of fumonisin B1, which inhibits ganglioside synthesis and, after three weeks, assayed the cultures for ganglioside content, for binding to tetanus toxin, and for several measures of toxin action. That fumonisin affords significant, although not total, protection against toxin action provides strong evidence that sialogangliosides are a functional component of the tetanus toxin receptor.

Differential Action of the Clostridial Neurotoxins at the Neuromuscular Junction. We have initiated studies to compare the relative effectiveness of the clostridial neurotoxins in blocking neurotransmission at the nerve-muscle synapse (primary site of botulinum neurotoxin action) with that at spinal cord synapses (primary site of tetanus neurotoxin action). We prepared nerve-muscle co-cultures in multicompartment culture chambers. These specialized preparations allow: physical segregation of the nerve-muscle synapse from the axons and from the cell bodies of the innervating neurons; electrical stimulation of axons passing under a barrier to enter the muscle compartment, and visual assessment of muscle twitch in response to stimulation as a measure of synaptic function. We also developed software for driving a motorized stage such that the locations of innervated muscle fibers can be stored and retrieved repeatedly. Previous data from spinal cord cell cultures indicated that tetanus toxin is twice as potent as BoNT A in blocking transmitter release; botulinum neurotoxins E and F have little effect in this system. In contrast, in the nerve-muscle system, botulinum neurotoxin A is more potent than tetanus toxin in blocking synaptic response. Botulinum neurotoxin F is even more potent than botulinum neurotoxin A, and the E serotype is less potent than the A serotype. Thus, these cultures reflect toxin actions *in vivo*, which are most likely to be due to receptor-related differences. The nerve-muscle preparations offer the potential for further studies of the cell biology of the toxins, including mechanisms for internalization and differential trafficking to their site of action.


Elaine Neale

Substrate Cleavage and Synaptic Blockade. The exact correspondence between extent of substrate cleavage and block in neurotransmitter release has not been analyzed carefully for the clostridial neurotoxins. In experiments using spinal cord cell cultures, we have shown, in collaboration with the Center for Biologics Evaluation and Research, FDA, that a complete block in transmitter release does not require cleavage of the entire substrate pool. With tetanus toxin and botulinum neurotoxins A and C, a 50% block in neurotransmission occurs after only about 25% of the respective substrate protein is cleaved. This suggests that the toxin might be sorted to the active zone in order to act on the relevant substrate pool; the mechanism for such sorting remains to be elucidated.

Selective Neurotoxicity of Botulinum Neurotoxin Type C. We completed our study of the effects of the C serotype of botulinum neurotoxin in spinal cord cell cultures. In the past, this serotype was not studied extensively, perhaps because it is not associated with human botulism and is not utilized for clinical

applications. This toxin has garnered recent attention, however, because it cleaves syntaxin, which has been shown to be associated with N-type calcium channels and to be involved in diverse non-neuronal exocytotic events. We demonstrated that, among the clostridial neurotoxins, the C1 serotype is unique in its neurotoxicity. With prolonged (up to five days) exposure, mature neurons exhibit severe degeneration, and neurons in newly plated cultures do not survive. Electron microscopy reveals the first clear signs of degeneration in synaptic terminals, i.e., accumulation of membranous sacs. Inhibiting axonal transport with colchicine does not prevent this accumulation, suggesting that the membranes arise from aberrant trafficking at the level of the synapse. Ultimately, the neurons undergo a retrograde degeneration. We have no evidence from electron microscopy for apoptosis; the signal that triggers the lethal necrosis is unknown. We believe that the toxin's proteolysis of syntaxin may affect not only synaptic vesicle fusion, but also may alter the activation state of syntaxin-associated calcium channels, allowing unregulated influx of calcium and causing death as a result of calcium overload. Experiments with ^{45}Ca support this hypothesis. These studies suggest that syntaxin plays a role in events critical not only for synaptic vesicle exocytosis but also for neuronal survival. Furthermore, this serotype may offer an advantage over the other serotypes for use in the therapy of muscle dystonias.

Synaptic Vesicle Membrane Recycling. Synaptic function is sustained over long periods because the synaptic vesicle pool is maintained by a recycling process involving the tight coupling of vesicle exocytosis with vesicle retrieval by endocytosis. Whereas all of the clostridial neurotoxins block synaptic vesicle exocytosis, nerve endings in cultures treated with botulinum neurotoxin A show unexpected but unequivocal evidence of synaptic vesicle membrane retrieval upon stimulation with an elevated concentration of potassium. The persistence of synaptic vesicle endocytosis has been demonstrated at the level of the light microscope by activity-dependent uptake of FM1-43, and at the level of the electron microscope by activity-dependent uptake of horseradish peroxidase into synaptic vesicles and into membranous intermediates in vesicle recycling. We have demonstrated for the first time that the endocytosis component of vesicle recycling is dependent on extracellular calcium. Further studies to determine why botulinum neurotoxin A does not completely block vesicle trafficking at the synapse should increase our understanding of the molecular mechanisms underlying synaptic vesicle membrane retrieval.

Neuronal Activity and the Development of Neuron Structure. The Section on Cell Biology also has a long-standing interest in the role of synaptic input on the development of neuronal structure. We have begun to analyze the number of synapses formed and the extent of axonal outgrowth in cultures exposed for one week to either tetrodotoxin (to block neuronal action potentials) or to tetanus neurotoxin (to block synaptic potentials). When the toxins are added within a few hours of plating, they have little effect on neuronal survival. Axons and synaptic terminals are visualized by fluorescence immunohistochemistry. We have developed software for the quantitation of synapse number based on a measure of total fluorescence. Preliminary data indicate that, depending on when the toxins are added after plating, neurons in electrically blocked cultures form a greater number of synapses than neurons that remain electrically active. This finding is consistent with the hypothesis that electrical activity is associated with synapse elimination. In other studies, we injected neurons with a fluorescent dye and analyzed dendrite structure for fractal dimension as a measure of branching complexity. The initial data show that neurons grown in the presence of tetrodotoxin are twice as complex as untreated neurons, primarily due to the presence of filamentous extensions along the dendrites. These data also support the idea that ongoing neuronal activity during development tends to simplify neuronal structure.

THE MELATONIN RHYTHM ENZYME

The Section on Neuroendocrinology has made important advances in understanding broad issues of the neural control of the biochemistry of cells, by studying the regulation of melatonin. Under the direction of ***David Klein***, these workers have focused on the key enzyme regulating melatonin production, serotonin N-acetyltransferase(AANAT). The levels of this enzyme exhibit large fluctuations that are regulated by neural signals. There is a 100-fold increase at night, and there is a very rapid disappearance of enzyme activity if animals are exposed to light in the middle of the night.

Work conducted in this laboratory has resulted in the discovery that, in all species examined, this increase in enzyme activity is always accompanied by an increase in total protein. This suggests that a highly conserved element in regulation regulates the rate of protein synthesis or the rate of protein degradation, or both.

Cyclic AMP Regulation of Transcription. Studies by this group have found that one factor contributing to this increase in some species is an increase in mRNA, which is most dramatic in the rat and mouse. In these rodents,

the increase in mRNA is about 150-fold. Studies on the promoter that regulates expression of the AANAT gene have revealed the presence of cyclic AMP binding protein response elements, which mediate these changes, and other studies have established that this increase is associated with an increase in cyclic AMP-dependent phosphorylation of the cyclic AMP binding protein CREB. The activity of this site is mediated by the competing binding protein ICER, which suppresses activation of the promoter. In addition, we have identified AP-1 sites, which may mediate negative regulation.

David Klein

A puzzling feature of this evidence of transcriptional regulation is that it is not seen in all species. For example, in sheep there is little or no change in mRNA, although protein levels change significantly. This suggests that other mechanisms of regulation are important in controlling AANAT protein.

Cyclic AMP Regulation of Proteosomal Proteolysis. An important advance in this line of research has been made in this laboratory, when it was established that the rapid decrease in enzyme activity seen at night is very closely related to proteolysis. This work is now determining the details of this proteolysis. The light- induced decrease in AANAT activity is seen in all species, and is thought to be mediated by highly conserved elements found in the AANAT molecules.

A Second Serotonin N-Acetyltransferase Gene. Our identification of a second and perhaps third AANAT gene in fish, which encode enzymes with distinctly different kinetics, tissue specificity, and substrate specificity is likely to expand our understanding of AANAT. It is expected that other AANAT genes will be found in all vertebrates. This adds a new and unexpected level of complexity to the interesting issue of how neural signals control biochemical events within cells.

MOLECULAR GENETICS OF NEUROTRANSMITTER DIVERSITY IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

The Unit on Molecular Control and Neurodifferentiation uses the enkephalin phenotype as a model to understand the molecular process by which the embryonic, multi-potent neuroglial progenitor cell becomes terminally-differentiated and committed to its final neurotransmitter phenotype. Neurons in the CNS communicate with each other and other target cells by one of the approximately 10 "classical" and one of approximately 30 "peptide" neurotransmitters such as enkephalin. As more and more evidence points toward the developmental origin of major neuropsychiatric and neurodegenerative disorders, it has become critical to understand fundamental developmental processes that lead to the normal, balanced phenotypic diversity in the adult CNS. The basal ganglia especially have been the target of intensive research, as a wide array of techniques (from molecular genetics to PET scan) demonstrated that it is the primary *"locus morbis"* of many disorders (e.g., autism, attention-deficit disorder, schizophrenia, Huntington's, and Parkinson's diseases). The main peptide neurotransmitter of the basal ganglia circuit is enkephalin, and its involvement in mediating pathological conditions such as autistic behavior, hallucinations in schizophrenia, personality changes in Huntington's disease, alcoholism, and drug abuse have been amply demonstrated.

Developmental-Specific Gene Regulation. We have been identifying novel DNA elements and their binding proteins that are critical for the developmentally regulated expression of the enkephalin (*ENK*) gene. The spatiotemporally coordinated and concerted protein-DNA interactions result in the well defined distribution of enkephalinergic neurons in the adult brain. We also study the role of far-distant DNA interactions and structural changes in the nuclear architecture during terminal neuronal differentiation and are searching for novel extra- and intracellular signaling mechanisms in the developing mammalian CNS.

Selective Repression of Neurotransmitter Genes in the Developing CNS. Our working hypothesis is that extracellular signals act as specifiers for the cellular phenotypes, including the enkephalin phenotype in the CNS. This early specification is followed by a whole cascade of intracellular events (execution) that, at the end of the developmental period, results in the proper expression pattern of phenotypic specifying genes, such as *ENK*, in the adult brain. Based on our preliminary results, this regulation is a progressive restriction of the *ENK*

gene expression which involves multiple interactions between various regulatory elements of the gene and DNA binding proteins, expressed in a spatially and temporally precisely defined manner in the developing CNS.

Novel Technique to Identify *Cis* and *Trans* Elements. We have developed a unique and unorthodox approach ("sample and probe") by combining microdissection of the developing brain regions with powerful molecular biological techniques, which permits us to study this developmental regulation at the level of protein-DNA interactions and nuclear structure and provides an unparalleled spatiotemporal resolution at the molecular level. Using this novel approach, we have identified several novel brain- and development-specific nuclear proteins. Of those, three have been investigated in detail.

New Family of Developmental Repressors. A novel cDNA encoding a developmental repressor of the rENK gene was isolated by a novel approach, in which a cDNA library made of neonatal brain was expressed in a phage display system. The phage cDNA library was incubated with biotinylated A/T-domain DNA and subsequently panned against streptavidin magnetic beads. Multiple rounds of panning, washing, elution, and phage amplification resulted in the isolation of a clone that is able to form a specific complex with radiolabeled A/T-domain in gel-shift assays. This clone, designated BAD-1 (Brain-, A/T- and Development-specific protein 1), encodes a novel 43 kDa protein. Homology search showed partial homology of BAD-1 with the cosmid L206D7 containing the human Huntington disease region. The DNA-binding domain of BAD-1 was found to be unusually enriched in hydrophobic residues. BAD-1 displays functional but not structural similarities to SATB1, the essential developmental regulator of thymocyte differentiation. BAD-1 is the first member of a novel family of brain and development-specific transcriptional regulators that recognize a specific sequence context and exhibit a high base pair unwinding propensity. *In vivo* and *in vitro* experiments, using the drug distamycin that specifically binds to the minor groove and thereby perturbs minor groove-protein interactions typical of nuclear matrix proteins, suggest that BAD-1 is a novel member of MAR-binding proteins that regulate gene expression by regulating chromatin structure.

Lineage Specification by Selective DNA Bending. A novel motif [TTTGCAT = Sept] on the rENK gene was identified as specific binding site for developmentally expressed proteins. The emergence of neuroepithelial precursors between ages E10-E14 is marked by a specific DNA-protein-complex, the P-complex (P for precursor). The entry of precursors into neuronal and glial lineages is marked by disappearance of the P-complex and appearance of two distinct complexes, the N-complex (N for neuro) and the G-complex (G for glia), whose expression patterns match the spatiotemporal pattern of neuro- and gliogenesis, respectively. These proteins are not recognized by antibodies raised against Oct-1 protein, and are not found outside the nervous system. A novel combination of UV and chemical cross-linking showed that the lineage-specific complexes (P, N and G) consist of three distinct proteins: precursor cells express only a 16.5 kDa protein that forms a homodimer and constitutes the basic DNA-binding unit; neuronal precursors express an additional 29 kDa protein that binds to the 33 kDa homodimer, and forms the N-specific 62 kDa complex; and glial precursors express a 23 kDa protein, which similarly binds to the 33 kDa DNA-binding homodimer to form the 56 kDa G-specific complex. Binding of the P-, G-, and N-specific complexes to the Sept-motif resulted in distinct, lineage-specific DNA bending ($P = 53°$, $G = 72°$, $N = 90°$), which may contribute to development-specific regulation of the motif-bearing genes such as the rENK gene.

Lymphoid-Specific Transcription Factor Specify Neurotransmitter Phenotype. A fragment located about 1500 bp upstream of the rENK gene was identified in our "sample and probe" screening as a site for development-specific protein-DNA interactions. Subsequent DNase I footprinting identified a binding site (GACGGGAGATCGCTCGT) that is similar to the site for a lymphoid-specific, developmentally regulated transcription factor, Ikaros, which regulates T cell commitment in developing thymocytes. We have shown that the Ikaros message is present in the developing but not in the adult brain and that the message is spliced in a brain-region and development-specific manner, resulting in distinct proteins with different DNA-binding ability.

GENE REGULATION BY NEURAL IMPULSES

Nervous system development is influenced by neural impulse activity in the prenatal and early postnatal periods. The Unit on Neurocytology and Physiology, under the direction of ***Douglas Fields***, is investigating the molecules involved in regulating the structure and function of the nervous system in response to appropriate patterns of neural activity, and is exploring the cellular mechanisms that regulate gene expression in response to neuronal firing. A combination of molecular, imaging, and electrophysiological techniques are employed on *in vitro* and *in vivo* preparations of mammalian neurons.

Activity-Dependent Regulation of Cell Adhesion Molecules. Our experiments to identify molecules that may participate in activity-dependent plasticity have shown that electrical activity of neurons in culture can influence the expression of neural cell adhesion molecules (CAMs). Cell adhesion molecules have a major influence on development of the nervous system. Our research is showing that expression of different CAMs is regulated by different patterns of impulse activity. For example, the cell adhesion molecule L1 is down-regulated by 0.1 Hz stimulation, but not 1 Hz stimulation in DRG neurons. N-cadherin is down-regulated by both frequencies of firing, and NCAM expression is not altered by either pattern of firing. Activity-dependent changes in CAM expression produce several functional and structural changes in neural networks in culture that could be important in remodeling the nervous system during development. Activity-dependent changes in adhesion and fasciculation of axons, association with glial cells, regulation of Schwann cell proliferation, and myelination are being investigated. Correlation between the effective stimulus patterns *in vitro* and activity patterns *in utero* suggests that the changes in impulse firing pattern experienced by a developing neural circuit could influence the developing structure and function of the nervous system.

Douglas Fields

Other research in the lab has revealed that neural impulse activity can regulate expression of N-cadherin in DRG neurons. This regulation operates at the level of transcription or stability of the mRNA transcripts. N-cadherin mRNA levels were decreased rapidly (within 24 hrs) by both 0.1 Hz and 1 Hz stimulation. These effects contrast with effects of impulse activity on expression of L1 and NCAM in terms of kinetics and sensitivity to specific frequencies of action potentials. These results show that genes for different cell adhesion molecules can be regulated by different frequencies of neural impulse activity. The mechanism by which the expression of CAMs is regulated by specific patterns of neural impulses is unknown.

Control of Myelination by Specific Patterns of Neural Impulses. Nervous system function in vertebrates is critically dependent on the formation of myelin sheaths surrounding central and peripheral axons during the perinatal period. Peripheral axons of dorsal root ganglion (DRG) neurons are myelinated by Schwann cells, and antibody blockade experiments have shown that initiation of myelination is dependent on the cell adhesion molecule L1. Abnormalities in myelination cause a wide range of neurological disorders due to defective impulse conduction, and the process of myelination and stages of psychological development are correlated in infants and children.

We have developed a preparation to study effects of axonal firing frequency on myelination of DRG axons by Schwann cells in culture. Compact myelin is identified by Sudan Black staining, immunocytochemical detection of myelin basic protein (MBP), and transmission electron microscopy. The ultrastructure of myelinated axons is normal in cultures stimulated at 0.1 or 1 Hz, but the number of myelinated profiles is reduced significantly in cultures that have been stimulated at 0.1 Hz compared with unstimulated axons or with axons firing at 1 Hz. Regulation of myelination and other axon-glial interactions by appropriate patterns of firing could be an important mechanism coordinating structure and function of myelinating axons.

Intracellular Signaling from Neural Impulses. It is known that expression of specific genes is necessary for persistent changes in the nervous system, but it is not clear how gene expression can be regulated by specific patterns of neural impulses. Our research indicates that the temporal dynamics of intracellular signaling cascades are critical in decoding different patterns of action potential activity. We are investigating the signaling mechanism by which action potentials of appropriate frequencies regulate expression of L1. To date, this research has included a broad survey of both intracellular and extracellular signaling molecules. Activation of second messengers, kinases, phosphatases, and transcription factors in the regulation of the immediate early gene c-*fos* is being studied in mouse DRG neurons to determine how information encoded in the temporal pattern of neural impulses is transduced and integrated within the cell to regulate expression of specific genes.

Using a combination of fluorescence imaging and confocal microscopy, molecular biology, biochemistry, and electrophysiology, we have measured the relation between neural impulse pattern, intracellular calcium transients, activation of mitogen-activated protein kinase (MAPK), the transcription factor cyclic AMP response

element binding protein (CREB), and induction of *c-fos* mRNA and Fos-beta-galactosidase in mouse primary DRG neurons. Bursts of action potentials separated by relatively long intervals (5 minutes) fail to activate the ERK MAP kinases in mouse DRG neurons, because the enzyme dephosphorylates in a shorter time than the interburst interval. Transcription factors dependent upon activation of this signaling pathway would not be responsive to this pattern of stimulation. In contrast, the transcription factor CREB is phosphorylated by brief bursts of action potentials, and CREB remains phosphorylated long after the stimulus is discontinued. Thus, either brief bursts of action potentials (1.8 s at 10 Hz) repeated frequently (1 min), or longer bursts of impulses (9 s at 10 Hz) repeated infrequently (5 min) activate CREB. Interactions between parallel signaling pathways exhibiting different dynamic properties further segregates temporal features of the stimulus. For example, maximal expression of *c-fos* results when action potentials are delivered in temporal patterns that co-activate both CREB and the ERK MAP kinase pathways. Results of these studies are consistent with the idea that the differing kinetics of parallel intracellular signaling reactions confer sensitivity to different temporal patterns of activation. In this way, distinct intracellular pathways controlling gene expression or other neuronal and synaptic functions are activated by distinct patterns of action potentials.

Long-Term Depression and Functional Synapse Elimination. Activity-dependent synapse elimination is likely to play a major role in the refinement of neuronal circuits in the mammalian neocortex during early postnatal development. We have successfully reproduced the process of long-term depression (LTD) of synapses in dissociated cortical cells in culture, and we are investigating the molecular mechanisms of synaptic reorganization in the visual cortex. In the past year, we have used whole-cell patch recordings from pairs of cortical neurons in culture to determine what activity patterns are necessary for the functional elimination of synaptic connections. By using a pattern that produces LTD in mature synapses (2 Hz), we found that the synaptic response could be reduced to undetectable levels with repeated stimulation at this frequency.

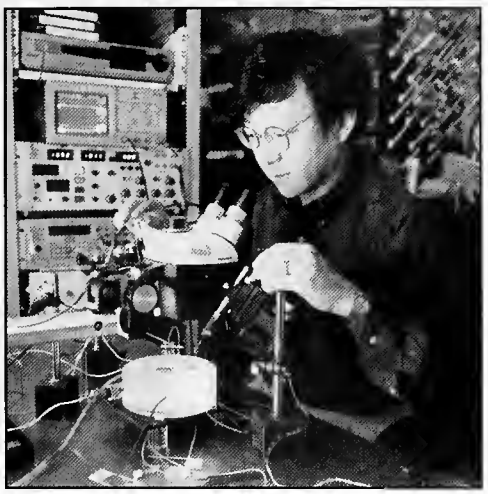
Role of Calmodulin in Growth Cone Motility and Intracellular Signaling. We are using a novel imaging technique to measure calmodulin (CaM) binding to its substrate in living neurons. This research should provide a better understanding of how a pivotal second messenger molecule, calmodulin (CaM), is activated in response to changes in cellular free calcium to regulate biological responses such as gene expression and neurite outgrowth. Our experiments are attempting to determine whether there are detectable changes in binding of CaM in the growth cone of elongating axons *in vitro* in response to depolarization-mediated calcium currents. The second experimental issue being addressed is how formation of a CaM-calcium complex in response to neuronal depolarization can lead to a change in gene expression. Such changes in gene expression are known to result from biochemical signaling cascades in which one of the earliest steps is the formation of CaM-calcium. It is not known, however, whether this complex is formed in cytoplasm or in the nucleus. Thus, following depolarization, either CaM-calcium activates signaling cascades in cytoplasm which are transmitted to the nucleus, or CaM-calcium itself enters the nucleus after depolarization.

MOLECULAR MECHANISMS OF SYNAPSE DEVELOPMENT AND PLASTICITY

The Unit on Synapse Development and Plasticity, led by ***Bai Lu***, investigates the molecular mechanisms that govern the formation of specific synapses in the nervous system. Recent efforts of this group focus on the function of neurotrophic factors in synapse development and plasticity. Neurotrophic factors are defined as secretory proteins that regulate long-term survival and differentiation of neurons. The role of neurotrophic factors in the structural integrity of the nervous system makes them attractive candidates as therapeutic agents for neurodegenerative diseases. However, recent studies by this group and other laboratories have revealed some unexpected, novel functions of neurotrophic factors in synapse development and plasticity. Of particular significance is our finding that the neurotrophin BDNF regulates the development of hippocampal long-term potentiation (LTP), a cellular mechanism for learning and memory. The group is now investigating the mechanisms by which BDNF regulates the hippocampal synaptic plasticity. In addition, the role of neurotrophic factors in the development of neuromuscular synapses is studied using a nerve-muscle co-culture system.

Presynaptic Modulation of Synaptic Transmission and Plasticity by BDNF in the Developing Hippocampus. Last year, we reported that the neurotrophin BDNF promotes the development of hippocampal LTP, a cellular mechanism for learning and memory. The BDNF effect on LTP was achieved by enhancing synaptic responses to high frequency, tetanic stimulation. We have now taken a number of approaches to demonstrate that BDNF regulates hippocampal LTP by presynaptic mechanisms. First, we showed that BDNF's effect on synaptic responses to repetitive stimulation depends strictly on the stimulation frequency, rather that on the number of stimulation pulses. BDNF only potentiates synaptic transmission when the hippocampal synapses are stimulated

at frequencies higher than 100 Hz. Under such high frequency stimulation, young hippocampal synapses undergo severe synaptic fatigue. Since it is known that synaptic fatigue induced by 100 Hz stimulation is due to a rapid depletion of the releasable pool of synaptic vesicles in presynaptic terminals, prevention of the fatigue by BDNF must be presynaptic in nature as well. Second, BDNF enhances paired pulse facilitation (PPF), the ratio of two EPSPs elicited by two successive stimulation pulses, at shorter interpulse intervals. A modulation of the ratio of PPF strongly indicates a change in transmitter release from the presynaptic terminals. Third, changes in the extracellular calcium concentrations alter the magnitude of the BDNF effect on tetanus-induced responses and PPF, suggesting that BDNF regulates the probability of neurotransmitter release. Finally, BDNF prevents synaptic fatigue and increases PPF in the presence of cyclothiazide, a drug that blocks non-NMDA receptor desensitization. Thus, the BDNF-induced increases in the HFS response are not likely to be due to an attenuation of non-NMDA receptor desensitization. In addition to above-mentioned evidence for presynaptic mechanisms for BDNF, we found that the BDNF effect on hippocampal LTP is confined to the tetanized synapses, and does not spread to neighboring synapses. The input specificity, high-frequency dependence, and presynaptic action of BDNF altogether provide a solid basis for the role of neurotrophins as retrograde messengers in the Hebbian model, which predicts that more active synapses are favored during synaptic competition. The activity-dependent synthesis and/or release of neurotrophins has been postulated to mediate the positive reinforcement. However, it has been difficult to fit neurotrophins into the Hebbian model, because neurotrophins would have a general enhancing effect on all synapses in the neighboring region exposed to the neurotrophins, regardless of whether a synapse is weak or strong. Our present finding suggests that BDNF preferentially enhances highly active synapses. Thus, even if the release of BDNF is not very localized at the active synapses, it could still favor more active synapses.



Bai Lu

Activity-Dependent Expression of NT-3 in Muscle Cells Mediates Synaptic Potentiation at the Developing Neuromuscular Junction. Our previous experiments indicate that the neurotrophin NT-3 promotes the maturation of neuromuscular synapse. Thus, treatment of the nerve-muscle co-cultures with NT-3 elicits changes in electrophysiological properties indicative of synapse maturation. Exogenous NT-3 also induces morphological changes and enhances the expression of the synaptic vesicle proteins in the presynaptic terminals. We have now examined whether the endogenous NT-3 may serve as a retrograde messenger at the neuromuscular junction. Retrograde messengers have long been thought to mediate activity-dependent modulation of synaptic efficacy. However, so far there is no definitive identification of any molecules as the retrograde messengers. There are a number of requirements that qualify a factor as a retrograde messenger. First, the factor has to be produced in the postsynaptic cells, and its receptor or effector has to be expressed in the presynaptic nerve terminals. Second, the expression or secretion of the factor should be activity-dependent. Third, the factor should act presynaptically to regulate synaptic efficacy. We tested whether neurotrophins can serve as retrograde messengers using the *Xenopus* neuromuscular junction as a simple model system to study retrograde messenger because the pre- and postsynaptic components are easily identifiable. The mRNA of TrkB receptors have been localized in presynaptic motoneurons. During early development, only BDNF and NT-3 are highly expressed in the postsynaptic muscle cells. We showed that membrane depolarization, elicited either by depolarizing agents or repetitive electric stimulation, rapidly and specifically increases the levels of NT-3 mRNA in developing muscle cells in culture. NT-3 gene expression is also enhanced by ACh, the neurotransmitter that causes muscle membrane depolarization. The effects of depolarization are mediated by increasing intracellular calcium concentration. Moreover, the depolarization-induced NT-3 potentiates synaptic activity at the developing neuromuscular synapses. Synaptic transmission at the neuromuscular synapses is markedly enhanced after treatment with conditioned medium from depolarized muscle cultures. This effect of conditioned medium is partially blocked by TrkC-IgG fusion protein, a specific scavenger of NT-3, suggesting that at least some of the released material is NT-3. Taken together, these results provide direct evidence that muscle-derived NT-3 may serve as a retrograde messenger for activity-dependent synaptic strengthening at the developing neuromuscular junction.

LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

Chief
Phillip G. Nelson, M.D., Ph.D.

Principal Investigators
Douglas E. Brenneman, Ph.D.
David C. Klein, Ph.D.
Y. Peng Loh, Ph.D.
Elaine A. Neale, Ph.D.

Tenure-Track Investigators
Andres Buonanno, Ph.D.
R. Douglas Fields, Ph.D.
Bai Lu, Ph.D.

Staff Scientist
Joanna Hill, Ph.D.

Senior Fellows
Denes v. Agoston, M.D., Ph.D.
Steven Coon, Ph.D.
Albert Dobi, Ph.D.
Alison Hickman, Ph.D.
Min Jia, M.D.
Keiko Mizuno, Ph.D.
Catherine Spong, M.D.
Chunfa Zhang, Ph.D.

Post-Doctoral Fellows
Ruben Baler, Ph.D.
Soledad Calvo, M.D., Ph.D.
Raquel Castellon, Ph.D.
Niamh Cawley, Ph.D.
Nelson Chong, Ph.D.
David Cool, Ph.D.
Roger Davenport, Ph.D.
Savita Dhavantari, Ph.D.
Serena Dudek, Ph.D.
Millicent Dugich, Ph.D.
Feleke Eshete, Ph.D.
Jonathan Gastel, Ph.D.
Gretchen Gibney, Ph.D.
Yoav Gothilf, Ph.D.
Wolfram Gottschalk, Ph.D.
Sunghee Kim, Ph.D.
Sau-Mei Leung, Ph.D.
Qun Li, M.D., Ph.D.
Francis Lim, Ph.D.
Alvin Lyckman, Ph.D.
Emmanuel Normant, Ph.D.
Chikara Oho, Ph.D.
John Sasner, Ph.D.
Michael Sasner, Ph.D.
Fu-Sheng Shen, Ph.D.
Pratap Venepally, Ph.D.

Pre-Doctoral Fellows
Heath Acuff
Neil Chheda
Jacquie Corness
Daphne Deurloo
Susan Lee
Vicki Olsen
Peter Rinaldi
Eric Snyder
Zehra Syed
Chang-Yu Wang
Zhe Zho

Senior Technical Specialists
Janet Hauser
Joan Weller

Guest Researchers
Haing Baik, M.D., Ph.D.
Susan McCune, M.D.
Maria Lazuna, Ph.D.
Min-Xu Li, M.D.
Aryan Namboodiri, Ph.D.
Andrew Parfitt, Ph.D.
Robert Weil, Ph.D.

ANNUAL BIBLIOGRAPHY

Baler R, Coon SL, Klein DC. Orphan nuclear receptor RZRβ: cyclic AMP regulates expression in the pineal gland. Biochem Biophys Res Commun 1996;220:975-8.

Baler R, Covington S, Klein DC.The rat arylalkylamine N-acetyltransferase gene promoter. cAMP activation via a cAMP-responsive element-CCAAT complex. J Biol Chem 1997;272:6979-85.

Bernard M, Klein DC, Zatz M. Chick pineal clock regulates serotonin N-acetyltransferase mRNA rhythm in culture. Proc Natl Acad Sci U S A 1997;94:304-9.

Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, Klein DC. Melatonin synthesis: photic and circadian regulation of serotonin *N*-acetyltransferase mRNA in the chicken pineal gland and retina. J Neurochem 1997;68:213-24.

Brenneman DE. The synapse: function, plasticity and neurotrophism. Rev J Chem Neuroanat 1997;12:221-2.

Brenneman DE, Hill JM, Gozes I, Phillips TM. Vasoactive intestinal peptide releases interleukin-1 from astrocytes. Ann N Y Acad Sci 1996;805:280-7.

Brenneman DE, Phillips TM, Festoff BW, Gozes I. Identity of neurotrophic molecules released from astroglia by vasoactive intestinal peptide. Ann N Y Acad Sci 1997;814:167-73.

Buonanno A, Rosenthal N. Molecular mechanisms regulating muscle plasticity. Dev Genetics 1996;19:95-107.

Calvo S, Stauffer J, Nakayama M, Buonanno A. Transcriptional control of muscle plasticity: differential regulation of troponin I genes by electrical activity. Dev Genetics 1996;19:169-81.

Chik CL, Liu QY, Li B, Klein DC, Zylka M, Kim DS, Chin H, Karpinski E, Ho AK. Alpha 1D L-type Ca(2+)-channel currents: inhibition by a beta-adrenergic agonist and pituitary adenylate cyclase-activating polypeptide (PACAP) in rat pinealocytes. J Neurochem 1997;68:1078-87.

Chodobski A, Loh YP, Corsetti S, Szmydynger-Chodobska J, Johanson CE, Lim Y-P, Monfils PR. The presence of arginine vasopressin and its mRNA in rat choroid plexus epithelium. Brain Res Mol Brain Res 1997;48:67-72.

Cool DR, Normant E, Shen F-S, Chen H-C, Pannell L, Zhang Y, Loh YP. Carboxypeptidase E is a regulated secretory pathway sorting receptor: Genetic obliteration leads to endocrine disorders in Cpe^{fat} mice. Cell 1997;88:73-83.

Coon SL, Mazuruk K, Bernard B, Roseboom PH, Klein DC, Rodriguez IR. The human serotonin *N*-acetyltransferase (EC 2.3.1.87) Gene: Structure, chromosomal localization and tissue expression. Genomics 1996;34:76-84.

Coon SL, McCune SK, Sugden D, Klein DC. Regulation of pineal alpha1B-adrenergic receptor mRNA: day/night rhythm and beta-adrenergic receptor/cyclic AMP control. Mol Pharmacol 1997;51:551-7.

Dibbern DA, Glazner GW, Gozes I, Brenneman DE, Hill JM. Inhibition of murine embryonic growth by HIV envelope protein and its prevention by vasoactive intestinal peptide and activity-dependent neurotrophic factor. J Clin Invest 1997;99:2837-41.

Erickson J, Weihe E, Schäfer MK-M, Neale E, Williamson L, Bonner TI, Tao-Cheng SJ-H, Eiden LE. The VAChT/ChAT "cholinergic gene locus": new aspects of genetic and vesicular regulation of cholinergic function. In: Klein J, Loffelholz E, eds. Proceedings of the Ninth International Symposium, Society for Cholingergic Mechanisms. New York: Elsevier, 1996;69-82.

Fields RD, Eshete F, Stevens B, Itoh K. Action potential-dependent regulation of gene expression: temporal specificity in Ca^{++}, CREB, and MAP kinase signaling. J Neurosci 1997;17;7852-66.

Friedman TC, Mastorakos G, Bouzas EA, Newman TD, Mullen NM, Horton EG, Costello R, Papadopoulos NM, Chrousos GP. Carbohydrate and lipid metabolism in endogenous hypercortisolism: shared features and metabolic syndrome X and NIDDM. Endocrine J 1996; 43:645-55.

Gao W, Dugich-Djordjevic MM, Weil RJ, Lu B. Therapeutical usage of neurotrophic factors: patent analysis. Expert Opin Therap Patents 1997;7:325-38.

Glazner GW, Yadav K, Fitzgerald S, Coven E, Brenneman DE, Nelson PG. Cholinergic stimulation increases thrombin activity and gene expression in cultured mouse muscle. Brain Res Dev Brain Res 1997;99:148-54.

Gozes I, Bardea A, Bechar M, Pearl O, Reshef A, Zamostiana R, Davidson A, Rubinraut S, Giladi E, Fridkin M, Brenneman DE. Neuropeptides and neuronal survival: neuroprotective strategy for Alzheimer's disease. Ann N Y Acad Sci 1997;814:161-6.

Gozes I, Brenneman DE. Activity-Dependent Neurotrophic Factor (ADNF): an extracellular neuroprotective chaperonin? J Mol Neurosci 1996;7:235-44.

Gozes I, Davidson A, Gozes Y, Mascolo R, Barth R, Warren D, Hauser J, Brenneman DE. Antiserum to activity dependent neurotrophic factor produces neuronal cell death in CNS cultures: immunological and biological specificity. Brain Res Dev Brain Res 1997;99:167-75.

Gozes I, Lilling G, Davidson A, Bardea A, Reshef A, Glazer R, Zamostiano R, Asjir-Fabian O, Ticher A, Ashkenazi IE, Moody TW, Rubinraut S, Fridkin M, Brenneman DE. Development of VIP agonists and antagonists with tissue and receptor specificity: effects of behavioral maturation, sexual function and the biologic clock. Ann N Y Acad Sci 1996;805:159-71.

Gressens P, Marret S, Hill JM, Brenneman DE, Gozes I, Fridkin M, Evrard P. Vasoactive intestinal peptide prevents excitotoxic death in the murine developing brain. J Clin Invest 1997;100:390-7.

Gressens P, Paindaveine B, Hill JM, Brenneman DE, Evrard P. Growth factor properties of VIP during early brain development. Ann N Y Acad Sci 1997;814:152-60.

Hill JM, Gressens P, Brenneman DE. Growth of the early postimplantation embryo: regulation by high affinity, GTP-insensitive VIP receptors. Ann N Y Acad. Sci. 1997;814:174-80.

Hill JM, McCune SK, Alvero RJ, Glazner GW, Brenneman DE. VIP regulation of embryonic growth. Ann N Y Acad Sci 1996;805:259-68.

Itoh K, Ozaki M, Stevens B, Fields RD.Activity-dependent regulation of N-cadherin in DRG neurons: differential regulation of N-cadherin, NCAM, and L1 by distinct patterns of action potentials. J Neurobiology 1997;33;735-48.

Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone M, Rodriquez IR, Begay V, Falcon J, Cahill G, Cassone VM, Baler R. The melatonin rhythm generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog Horm Res 1997;42:307-55.

Klein DC, Roseboom PH, Coon SL. New light is shining on the melatonin rhythm enzyme: the first post cloning view. Trends Endocrinol Metabol 1996;7:106-12.

Kuenzel WJ, McCune SK, Talbot RT, Sharp PJ, Hill JM. Sites of gene expression for vasoactive intestinal peptide (VIP) throughout the brain of the chick (*Gallus domesticus*). J Comp Neurol 1997;381:101-18.

Lecchi P, Loh YP, Snell CR, Pannell LK. The structure of synenkephalin (pro-enkephalin $_{1-73}$) is dictated by three disulfide bridges. Biochem Biophys Res Commun 1997:232:800-5.

Li MX, Jia M, Fields RD, Nelson PG. Modulation of calcium currents by electrical activity. J Neurophysiol 1996;76:2595-607.

Loh YP, Shen F-S, Snell CR, Normant E, Cool DR. Receptor-mediated targeting of hormones to secretory granules: role of Carboxypeptidase E. Trends Endocrinol Metab 1997;8:130-6.

Lu B, Figourov A. Role of neurotrophins in synapse development and plasticity. The Neuroscientist 1997;8:1-12.

Lu B, Gao W. The use of neurotrophic factors as novel therapeutic agents for diseases in the nervous system. In: Zhou H, Diasio RB, Zhang RW, eds. Principles and practice of pharmaceutical research and development in the Western countries: Health Press. Beijing, China. 1997.

Lu B, Papov P, Czernik A, Wang T, Poo MM, Greengard P. Expression of synapsin I reflects maturation of neuromuscular synapse. Neuroscience 1996; 74:1087-97.

Nelson PG, Fitzgerald S, Rapoport SI, Neale EA, Galdzicki Z, Dunlap V, Bowers L, Agoston Dv. Cerebral cortical astroglia from the trisomy 16 mouse, a model for Dawn syndrome, produce neuronal cholinergic deficits in cell culture. Proc Natl Acad Sci USA 1997;94:12644-8.

Olafsson P, Soares HD, Wang T, Herzog KH, Morgan JI, Lu B. The Ca^{2+} binding protein frequenin is a nervous system-specific protein preferentially localized in neurites. Brain Res Mol Brain Res 1997;44:73-82.

Ozaki M, Sasner M, Yano R, Lu H, Buonanno A. Neuregulin induces expression of an NMDA receptor subunit. Nature 1997;390:691-4.

Roseboom PH, Coon SL, Baler R, McCune SK, Weller JL, Klein DC. Melatonin synthesis: regulation of the more than 150-fold nocturnal increase in serotonin N-acetyltransferase messenger RNA in the rat pineal gland. Endocrinology 1996;137:3033-44.

Sasner M, Buonanno A. Distinct N-methyl-D-aspartate receptor 2B subunit gene sequences confer neural and developmental specific expression. J Biol Chem 1996;271:21316-22.

Shen F-S, Loh YP. Intracellular misrouting and abnormal secretion of adrencorticortropin and growth hormone in Cpe^{fat} mice associated with a carboxypeptidase E mutation. Proc Natl Acad Sci USA 1997;94:5314-9.

Shen L, Figourov A, Lu B. Recent progress in studies of neurotrophic factors and their clinical implications. J Mol Med 1997;75:6737-44.

Sugden D, Anwar N, Klein DC.Rat pineal alpha 1-adrenoceptor subtypes: studies using radioligand binding and reverse transcription-polymerase chain reaction analysis. Br J Pharmacol 1996;118:1246-52.

Weller JL, Jaffee H, Roseboom PH, Zylka M, Klein DC. 2D-PAGE analysis: adrenergically regulated pineal protein AIP 37/6 is a phosphorylated isoform of cytosolic malate dehydrogenase. Brain Res 1996;713:8-16.

Xie K, Wang T, Olafsson P, Mizuno K, Lu B. Activity-dependent expression of NT-3 in muscle cells in culture: implications in the development of neuromuscular junctions. J Neurosci 1997;17:2947-58.

LABORATORY OF EUKARYOTIC GENE REGULATION

Alan G. Hinnebusch, Ph.D., Chief

TRANSCRIPTIONAL AND TRANSLATIONAL REGULATORY MECHANISMS IN NUTRIENT CONTROL OF GENE EXPRESSION

Yeast cells respond to starvation for any single amino acid or for purines by the global activation of genes encoding enzymes involved in amino acid and purine biosynthesis; this regulatory mechanism is termed general amino acid control. Members of the Section on Nutrient Control of Gene Expression, led by ***Alan Hinnebusch***, have elucidated the molecular mechanisms responsible for several aspects of this general control response through a combination of genetic and biochemical studies. We have shown that the major "choke-point" in the system is translational induction of GCN4, a transcriptional activator of the coregulated biosynthetic genes. Genetic and molecular analysis of this translational control mechanism have led to detailed investigations of several steps in the assembly of the translation initiation complex, particularly those influencing the binding of initiator tRNA to the ribosome. The analysis of mutations affecting *GCN4* translation has identified the subunits of translation initiation factors eIF2, eIF2B, a protein kinase that regulates their activities, and two nuclear factors involved in the maturation of initiator tRNA. The analysis has also provided molecular insights into the mechanism whereby phosphorylation of eIF2 leads to down-regulation of translation under stress or starvation conditions, and different modes of regulating the eIF2α kinases. This group is also investigating the mechanism of transcriptional activation by GCN4, focusing on the structure of the activation domain and its physical interactions with transcriptional coactivators. Finally, we have probed the molecular basis for purine repression of adenine biosynthetic genes, and found that complex formation between a Myb-related DNA binding protein (BAS1) and a homeodomain transcriptional activator (BAS2) is a key regulated step in the transcription of these genes.

Translational Control by Phosphorylation of eIF2 and Inhibition of the Guanine Nucleotide Exchange Factor eIF2B. GCN4 expression is repressed at the translational level by four short upstream open reading frames (uORFs) in the mRNA leader. In cells starved for an amino acid or purine, ribosomes bypass the uORFs and translate *GCN4* instead, because they fail to bind to the ternary complex consisting of eIF2, GTP, and charged initiator $tRNA^{Met}$ until after scanning past uORF4. This occurs because the concentration of ternary complexes is reduced in starved cells by phosphorylation of the α subunit of eIF2 by the protein kinase GCN2. Previously, we obtained several lines of evidence indicating that phosphorylation of eIF2 converts it from a substrate to a competitive inhibitor of its guanine nucleotide exchange factor, eIF2B. A large number of point mutations were isolated in the GCN3, GCD7, and GCD2 subunits of eIF2B that suppress the effects of eIF2 hyperphosphorylation without affecting the catalytic activity of eIF2. These subunits are similar in sequence, and the regulatory mutations map in two approximately 70-amino-acid clusters located in similar positions in all three proteins. We showed that these mutations do not affect the stability of eIF2B nor do they exclude GCN3, the only dispensable subunit, from the eIF2B complex. We proposed that GCD2, GCD7, and GCN3 comprise a regulatory subdomain, in which homologous segments form a surface that interacts with residues in eIF2α surrounding the phosphorylation site and that these segments mediate the inhibition of the nucleotide exchange reaction with phosphorylated eIF2. Because the majority of eIF2 is phosphorylated in several regulatory mutants without any detectable inhibition of growth, we suggested that these mutant forms of eIF2B are able to accept phosphorylated eIF2 as a substrate.

Biochemical evidence has been obtained confirming this proposed mechanism for regulatory mutations in the *GCN3* and *GCD7* eIF2B subunits. We developed a plasmid system for overexpression and affinity-purification of eIF2, to allow biochemical analysis of the effects of eIF2B regulatory mutations on nucleotide exchange using phosphorylated eIF2 as the substrate. We found that deletion of *GCN3* and two point mutations in *GCD7*, all of which suppress the toxicity of eIF2α phosphorylation *in vivo*, allow eIF2B to catalyze nucleotide exchange on phosphorylated and unphosphorylated eIF2 with equal efficiencies.

Our model of eIF2B regulation is also supported by the previous finding that overexpression of GCD2, GCD7, and GCN3 *in vivo* leads to formation of a stable eIF2B subcomplex that can be immunopurified from cell extracts. Overexpression of this subcomplex overcomes the inhibition of protein synthesis by phosphorylated

eIF2 *in vivo*, and it was suggested that the GCD2/GCD7/GCN3 regulatory subcomplex can sequester eIF2(αP) and rescue the recycling of nonphosphorylated eIF2 by the native 5-subunit eIF2B. Biochemical support for this hypothesis has been obtained by showing that the GCD2/GCD7/GCN3 subcomplex can bind to eIF2 *in vitro* and that the subcomplex interacts more tightly with the phosphorylated form of the protein. The remaining two eIF2B subunits GCD1 and GCD6 can also form a stable subcomplex that interacts with eIF2; however, it binds equally well to phosphorylated and unphosphorylated eIF2. Remarkably, the GCD1/GCD6 subcomplex has full eIF2B catalytic activity that is unaffected by eIF2 phosphorylation. These results establish that eIF2B contains two independent binding sites for eIF2, which mediate its catalytic and regulatory functions. The GCD1/GCD6 subcomplex contains the active site but is insensitive to phosphorylation of the substrate eIF2. The GCD2/GCD7/GCN3 subcomplex has no catalytic activity but can sense the phosphorylation status of eIF2 and block the function of the GCD1/GCD6 subcomplex when eIF2 is phosphorylated. The eIF2B regulatory mutations do not appear to reduce binding of phosphorylated eIF2, but instead abrogate the inhibitory effect of the GCD2/GCD7/GCN3 subcomplex on the catalytic subcomplex when phosphorylated eIF2 is bound.

Identification of a Nuclear Complex Containing GCD10 and GCD14 Involved in the Maturation of Initiator $tRNA_i^{Met}$. Mutations in *GCD10* have the same effects on *GCN4* expression and general translation as mutations impairing subunits of eIF2 or eIF2B. Previously, we showed that the *GCD10* protein is essential for translation initiation *in vivo*, and in collaboration with John Hershey's laboratory, we found that GCD10 is identical to a 54 kDa RNA-binding protein that copurifies with eIF3 activity. Combining the results of biochemical studies on mammalian eIF3, and the effect of *gcd10* mutations on *GCN4* translation, we proposed that binding of ternary complexes to 40S subunits is impaired in *gcd10* mutants. This hypothesis was consistent with our isolation of all four *IMT* genes encoding $tRNA_i^{Met}$ as dosage suppressors of the *gcd10* mutations, assuming that elevated levels of $tRNA_i^{Met}$ would raise the levels of the ternary complex and compensate for decreased efficiency of ternary complex binding to the ribosomes.

Alan Hinnebusch

We have now shown that steady-state levels of $tRNA_i^{Met}$ are substantially reduced in *gcd10* mutants, due to increased turnover, and that the high-copy *IMT* genes simply restore wild $tRNA_i^{Met}$ levels in the mutants. In fact, *GCD10* can be deleted in strains overexpressing $tRNA_i^{Met}$, indicating that accumulation of this tRNA is the essential function of *GCD10*. Mutations in the *GCD14* gene were found to impair processing of $tRNA_i^{Met}$ and GCD10 and GCD14 were shown to coimmunoprecipitate and copurify. Substantial amounts of both GCD10 and GCD14 were localized to the nucleus by immunofluorescence. These findings indicate that GCD10 and GCD14 reside in a complex that functions in the nucleus in the maturation of $tRNA_i^{Met}$. The reduction in mature cytoplasmic $tRNA_i^{Met}$ in *gcd10* and *gcd14* mutants can account for their effects on *GCN4* translation.

Characterization of eIF3 and its Role in Assembly of the Preinitiation Complex for Protein Synthesis. Using a polyhistidine-tagged form of PRT1, the 90 kDa subunit of yeast eIF3, we have carried out affinity-purification of eIF3 and used mass spectrometry of tryptic peptides to identify the four polypeptides that are stably associated with PRT1. Remarkably, they correspond to the only five proteins encoded in the yeast genome with strong similarity to any of the nine subunits of human eIF3, suggesting that they comprise a conserved eIF3 complex. Direct interactions among three of the yeast subunits PRT1, TIF34, and TIF35 has been demonstrated by *in vivo* and *in vitro* interaction assays using recombinant proteins, and the domains mediating these interactions have been mapped in each of three proteins. TIF34 belongs to the WD-repeat family of proteins and is expected to adopt the β-propeller structure of β-transducin. Point mutations that weaken the *in vitro* interaction between TIF34 and the PRT1 and TIF35 subunits were identified in the sixth and seventh WD repeats of TIF34. The temperature-sensitive lethal phenotypes of these mutations can be suppressed by overexpression of *TIF35*, confirming the importance of these interactions for eIF3 function *in vivo*.

Interestingly, a large fraction of eIF5 copurified with the His-tagged eIF3 complex, indicating a novel interaction between these two initiation factors. eIF5 stimulates GTP hydrolysis in the eIF2/GTP/Met-$tRNA_i^{Met}$ ternary complex, catalyzing release of $tRNA_i^{Met}$ to the mRNA-40S ribosome complex. Results of *in vivo* interaction

assays indicate that eIF5 binds to the core subunit of eIF3 encoded by *NIP1*, and to the β subunit of eIF2. NIP1 was also found to interact with the *SUI1* protein, which has been linked genetically to eIF5. It is known that eIF3 stably binds to 40S ribosomal subunits in the absence of other initiation factors. Taken together, the data suggest that eIF3 recruits eIF5 and SUI1 to the 40S preinitiation complex, and that eIF5, in turn, stabilizes the binding of the eIF2/GTP/Met-tRNA$_i^{Met}$ ternary complex to the ribosome, in addition to stimulating the GTPase activity of eIF2. These findings provide important new insights into the molecular interactions involved in the assembly and function of the translation initiation complex.

Role of Autophosphorylation and Protein Dimerization in Activation of GCN2 Kinase Function. GCN2 contains regulatory domains both N-terminal and C-terminal to its protein kinase (PK) catalytic domain, including a region related to histidyl-tRNA synthetase (HisRS), a ribosome-binding domain, and a degenerate kinase domain. The HisRS-related domain can bind to tRNA *in vitro* and is believed to mediate the stimulation of GCN2 kinase activity by uncharged tRNA present in amino acid-starved cells. Using *in vivo* interaction assays and *in vitro* protein-binding experiments, we have found that the isolated PK can dimerize with the C-terminal domains, that the PK domain can interact with the pseudokinase, HisRS, and ribosome-binding domains, and that the HisRS and ribosome-binding domains also interact with one another. Dimerization of full-length GCN2 has been confirmed by coimmunoprecipitation from cell extracts, and it was shown that the ribosome-binding domain is the region most critically required for dimerization. The fact that *gcn2* alleles with mutations in the kinase domain interfere with wild-type *GCN2*, and that this dominant-negative phenotype requires the ribosome-binding/dimerization domain to be present in the mutant allele, provides evidence that GCN2 functions as a dimer *in vivo*. We speculate that interactions between the PK, HisRS, and C-terminal domains mediate conformational transitions in GCN2 involved in stimulating kinase activity in response to binding of uncharged tRNA to the HisRS domain.

For catalytic activity, several protein kinases are known to require phosphorylation on conserved Thr or Ser residues located between kinase subdomains VII and VIII. We found that Thr-813 and Thr-818 in GCN2 are autophosphorylated *in vitro* and that these residues are also required for *GCN2* function *in vivo*. We conclude that autophosphorylation on these Thr residues is an important step in GCN2 activation. Interestingly, we also showed that Thr residues in the human eIF2α kinase PKR (Thr-446 and Thr-451) at the exact positions corresponding to Thr-813 and Thr-818 in GCN2 are required for PKR kinase function in both yeast and mammalian cells; using mass spectrometry, we proved that PKR autophosphorylates on Thr-446 *in vivo*. Thus, autophosphorylation in the activation loop is an important step in kinase activation conserved between yeast and human eIF2α kinases.

Evidence that GCN1 and GCN20 Function on the Ribosome as Activators of the eIF2α Kinase GCN2. We showed previously that the GCN1 and GCN20 proteins are components of a high molecular weight complex required *in vivo* for phosphorylation of eIF2α by GCN2, and that an N-terminal segment of GCN20 and an internal segment of GCN1, which is closely related to translation elongation factor 3 (EF3), mediate complex formation between the two proteins. GCN20 also contains a region highly similar to EF3 comprising two ATP-binding cassettes. By immunofluorescence experiments, we showed that GCN1 and GCN20 are distributed throughout the cytoplasm; biochemical fractionation studies indicated that both proteins bind to translating 80S ribosomes, which is consistent with the hypothesis that GCN1 and GCN20 function at the ribosomal A site to detect uncharged tRNA and mediate the activation of ribosome-bound GCN2. We speculate that GCN1/GCN20 mediates that binding of uncharged tRNA to the acceptor (A) site, or facilitates its release from the A-site and its delivery to the HisRS domain of.GCN2. We have identified human homologs of GCN1 and GCN20, and a human version of GCN2 has been reported by others, suggesting that phosphorylation of eIFα in response to amino acid limitation is a regulatory mechanism conserved between yeast and humans.

Mechanism of Transcriptional Activation by GCN4. In addition to studying translational control of *GCN4*, we have been investigating how the GCN4 protein activates transcription. Our previous mutational analysis showed that the GCN4 activation domain contains seven clusters of bulky hydrophobic amino acids distributed throughout the N-terminal half of the protein that can function in different combinations to activate transcription *in vivo*. Point mutations in the hydrophobic clusters have been used to demonstrate specific interactions between the GCN4 activation domain and subunits of several transcriptional coactivators. These include three TAF$_{II}$ proteins found associated with the TATA-binding protein in transcription factor IID, SRB proteins found in the mediator complex associated with RNA polymerase II holoenzyme mediator, and ADA proteins found associated with the histone deacetylase GCN5. Our findings suggest that GCN4 is capable of recruiting each of these coactivators to the promoter, providing redundant means of stimulating the assembly of a transcription

initiation complex. In the course of these studies, we obtained evidence for a composite complex containing components of TFIID and the ADA/GCN5 coactivator. We speculate that GCN4 may interact with the TAF_{II} proteins only in the context of this composite complex. This work paves the way for the identification of specific subunits of the mediator and ADA/GCN5 coactivator complexes that interact directly with the GCN4 activation domain.

Transcriptional Control of Adenine Biosynthetic Genes. The mechanism of repression of the transcription of adenine biosynthetic genes under conditions of excess adenine has also been examined. Previously, a 67 bp fragment of the *ADE5,7* promoter was shown to be an adenine-repressible enhancer that contains three critical elements of 6-10 nt in length that function as the binding sites for the BAS1, BAS2, and ABF1 transcriptional activators. By studying fusions between BAS1 and BAS2 and the bacterial DNA-binding protein LexA, we provided evidence that BAS1 and BAS2 must physically interact at the promoter to activate transcription. Mutational analysis indicated that BAS1 contains a potent activation domain that is masked by inhibitory segments in the protein, and this activation function is unmasked by complex formation with BAS2. Because overexpressing BAS2 allows BAS1 to activate transcription more effectively under repressing conditions, we surmised that complex formation between BAS1 and BAS2 is down-regulated by adenine. Taken together, our results show that interaction between BAS1 and BAS2 is crucial for generating a potent transcriptional activator, and that inhibition of BAS1/BAS2 complex formation is a key step in the repression of *ADE* gene transcription.

TRANSPOSITION OF RETROELEMENTS IN FISSION YEAST

Retroelements including retroviruses and retrotransposons are widely distributed in nature. The great importance of these elements in biology in general makes it imperative to take advantage of the possibilities offered by yeast molecular genetics in their study. The Unit on Eukaryotic Transposable Elements, led by ***Henry Levin***, focuses on the mechanisms of retroelement replication and integration into the host genome. Our approach to understanding the complex interactions between the retroelement and its host is to study retrotransposons, a family of elements that are closely related to retroviruses. A significant advantage to studying retrotransposons is that they exist in hosts, such as the yeast *Schizosaccharomyces pombe*, that can be studied using sophisticated molecular genetic techniques.

Self-Priming Mechanism of the Tf1 Retroelement of Fission Yeast. We previously obtained strong genetic evidence that the transposon Tf1 uses a novel self-priming mechanism to initiate cDNA synthesis, in contrast to the tRNA-priming mechanisms used by other retroelements. We previously provided genetic evidence that the first 11 nucleotides of the Tf1 transcript anneal to the primer binding site by showing that base-pair complementarity between these two sequences is essential for transposition and reverse transcription *in vivo*. We obtained biochemical evidence to support for the mechanism by showing that Tf1 mRNA is cleaved between the 11th and 12th nucleotide, liberating the primer from the rest of the transcript. The latter involved making use of Tf1 mutations generated in the active site of reverse transcriptase to trap the priming intermediates, and a "splint ligation" assay to prove the existence of Tf1 transcripts lacking the first 11 nucleotides. Together, these data strongly support a model in which Tf1 priming involves cleavage of the first 11 nucleotides of the Tf1 transcript, followed by reverse transcription from the 3′ hydroxyl of the cleaved transcript annealed at the primer binding site. Recently, we established that complementarity between the 11th nucleotide of the primer and the PBS is critical for reverse transcription.

Henry Levin

Analysis of a large set of point mutations near the primer binding site previously revealed the existence of a complex RNA structure, composed of 27 base pairs, that includes the primer binding site and the first 11 nucleotides of the Tf1 transcript. Mutational analysis indicated that base complementarity in the predicted helical regions of this structure is essential for transposition and reverse transcription *in vivo*. We have identified an additional structure composed of a seven-base-pair stem and a 25-nucleotide loop and have shown by extensive mutagenesis experiments that this stem-loop is required for cleavage of the Tf1 transcript, reverse transcription, and transposition *in vivo*. The

size and function of this stem-loop and its position just upstream of the primer binding site in Tf1 is analogous to the U5-IR stem-loop of Rous sarcoma virus. The U5-IR stem-loop has also been identified in several other retroviruses suggesting that this structure has been highly conserved in the evolution of retroelements.

Genetic Analysis of Host Genes Involved in Tf1 Transposition. In an effort to identify host factors that contribute to the transposition of Tf1, we have isolated mutant strains of *S. pombe* that are defective for transposition. Mutations in three genes, *hop1*, *hop2*, and *hop3*, were identified that greatly reduce the frequency of Tf1 transposition. The *hop3* and *hop5* mutants exhibit normal levels of Tf1 protein expression and reverse transcript production, suggesting a defect in the integration of the Tf1 reverse transcript into the host genome. With the aid of large-scale complementation screens, *hop3* and *hop5* have now been cloned from *S. pombe* genomic libraries. The sequence of the *hop3*-encoded protein shows strong similarity to that of the *SIN3*-encoded proteins found in *S. cerevisiae* and mammals. Because SIN3 proteins are known to contribute to the deacetylation of histones and the condensation of chromatin, we think it possibile that Tf1 integration requires histone deacetylation to generate a more open chromatin structure at the integration site. This idea is supported by the finding that an inhibitor of histone deacetylases, trichostatin, inhibits Tf1 transposition *in vivo* to the same extent as does the *sin3/hop3* mutation. The sequence of the *hop5* protein indicates a close relationship with a class of evolutionarily conserved proteins involved in the import of proteins into the nucleus, known as nucleoporins. The *hop5* protein is most closely related to the class of nucleoporins that contain multiple copies of FXFG repeats. We speculate that *hop5* encodes a nucleoporin in *S. pombe* that is particularly important for transit into the nucleus of the Tf1 preintegration complex containing integrase and reverse transcripts. This work on the *hop* genes is yielding new insights into the complex molecular interactions between retroelements and their cellular hosts.

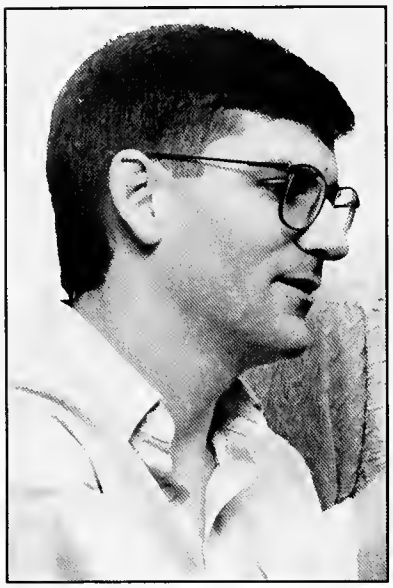
STRUCTURE AND REGULATION of eIF2α KINASES AND A NOVEL PATHWAY FOR TRANSLATION INITIATION INVOLVING A BACTERIAL IF2 HOMOLOG

The Unit on Protein Biosynthesis, headed by ***Thomas Dever***, is seeking to identify the structural determinants of substrate recognition by the eIF2α kinases, and to illuminate the cellular and viral mechanisms employed to control eIF2 function by regulation of these kinases. By delivering initiator $tRNA^{Met}$ to the ribosome, eIF2 plays a pivotal role in the initiation of protein synthesis, and it also functions in recognition of the AUG start codon during the scanning process. Therefore, it undoubtedly interacts with many other initiation factors and with the ribosome during the course of initiation. Down-regulation of eIF2 activity by phosphorylation of its α subunit is a highly conserved mechanism for reducing the rate of general protein synthesis under conditions of starvation and stress in mammalian cells, and it mediates gene-specific translational induction of *GCN4* expression in yeast. The human eIF2α kinase PKR has growth suppressive properties, and its induction is an important aspect of the interferon-mediated antiviral response. In addition, PKR has been implicated as a tumor suppressor in mammalian cells. It is appropriate, therefore, to exploit the powerful tools of yeast molecular genetics to probe all aspects of the structure, function, and regulation of eIF2, its protein kinases, and the regulators of these kinases. In addition, we are exploring a yeast protein that is homologous to bacterial initiation factor 2, can functionally substitute for mammalian eIF2 in *in vitro* reactions, and which is required in yeast cells for general translation initiation and the specialized reinitiation process involved in translation of *GCN4* mRNA.

Molecular Determinants of Substrate Recognition by eIF2α Kinases Identified Using a Pseudosubstrate Inhibitor. To identify amino acids in eIF2α that are critically required for its recognition by eIF2α kinases, we have carried out a mutational analysis of the K3L protein, a pseudosubstrate inhibitor of PKR encoded by *Vaccinia* virus. We established a system for suppressing the growth inhibitory effects of PKR in yeast cells by co-expressing the K3L protein, and using this system we identified both loss-of-function and hyperactive K3L mutations. One of the hyperactive mutations makes K3L more similar to eIF2α at a residue adjacent to the phosphorylation site in eIF2α at position 51. Loss-of-function K3L mutations reveal that the amino acid sequence KGYID, located in the C-terminal portion of K3L and identical to residues 79-83 in eIF2α, is critical for K3L function. Both *in vitro* protein-binding and *in vivo* protein-interaction assays demonstrate that these K3L mutations alter the affinity of K3L for PKR. In addition, mutation of Tyr-81 in the KGYID sequence in eIF2α reduces phosphorylation of the protein by GCN2 *in vivo*. These results strongly suggest that protein contacts over 30 residues from the site of phosphorylation in eIF2α are important for its recognition by an eIF2α kinase. Glu-49 and Leu-50, immediately preceding the phosphorylation site at Ser-51, are also critically required for

phosphorylation by GCN2 *in vivo*, indicating that kinase recognition of eIF2α requires contacts both near (Glu-49) and far (Tyr-81) from the phosphorylation site.

In an effort to map molecular determinants of substrate recognition by PKR, we isolated numerous PKR point mutations that confer resistance to inhibition by K3L. Interestingly, all of the mutations cluster in the C-terminal lobe in a region corresponding to the binding site for a pseudosubstrate inhibitor of protein kinase A. This region is likely to be required for substrate binding to PKR.



Tom Dever

A Novel Regulator of eIF2α Kinases. In searching the databases for other potential regulators of PKR, we discovered a protein encoded by *Baculovirus* that appears to be a truncated kinase domain, most similar in sequence to the eIF2α kinases. We have shown that expression in yeast cells of this protein, called pk2, can inhibit phosphorylation of eIF2α by both GCN2 and PKR, suggesting that insect cells contain an eIF2α kinase that is antagonized by pk2 during virus infection. Supporting this notion, we demonstrated that phosphorylation of eIFα is reduced and initiation factor eIF2B activity is increased in insect cells infected with *Baculovirus* expressing pk2. To show that down-regulation of eIF2α phosphorylation by pk2 during an infection can stimulate virus propagation, we showed that expression of PKR in insect cells reduces the yield of a pk2-deleted virus compared with wild-type virus and that propagation of the pk2-deficient virus can be rescued by expressing pk2 in *trans*. We speculate that pk2 can protect *Baculovirus* from the negative effects of eIF2α phosphorylation in the wild under stress or starvation conditions where eIF2α kinases become activated. We have demonstrated by coimmunoprecipitation experiments that pk2 can form a stable complex with PKR when the proteins are coexpressed in yeast cells. Interestingly, the corresponding C-terminal fragment of eIF2α kinase GCN2 also functions as a dominant inhibitor of wild-type GCN2 and PKR in yeast. These results establish a novel mechanism for inhibition of a protein kinase by formation of an inactive heterodimer between the kinase and a polypeptide resembling the kinase catalytic lobe.

Tyrosine Phosphorylation by PKR *in Vivo*. To date, the only proven substrate of PKR is eIF2; however, there are indications that other proteins may be phosphorylated by PKR in human cells, and the mouse homolog of PKR (TIK) was first identified in a screen for tyrosine kinases. We have shown that PKR and HRI, a second mammalian eIF2α kinase, can phosphorylate eIF2α on a Tyr residue at position 51. These results show that PKR is a dual-specificity kinase, and they raise the interesting possibility that PKR may phosphorylate additional substrates on tyrosine in human cells that could be involved in growth regulation or signal transduction.

Function of an IF2-Homolog in Yeast Translation Initiation. Yeast encodes a protein called FUN12 that is related to the prokaryotic translation initiation factor IF2. In bacterial cells, IF2 stimulates binding of initiator $tRNA^{Met}$ to ribosomes, the same function carried out by eIF2 in conventional translation initiation in eukaryotic cells. We are investigating the possibility that FUN12 replaces eIF2 in an alternative initiation mechanism in eukaryotic cells. Although FUN12 is nonessential for yeast survival, it is required for efficient translation of many cytoplasmic mRNAs and for normal cellular growth. The deleterious effects of a *fun12* deletion can be partially suppressed by overexpressing initiator $tRNA^{Met}$, or by increasing the concentration of free 40S ribosomal subunits by deletion of a 60S ribosomal protein (L16). These genetic findings support the idea that FUN12 functions in the delivery of initiator $tRNA^{Met}$ to 40S ribosomes. Strong biochemical evidence supporting this conclusion was provided by the finding that recombinant FUN12 can fully substitute for mammalian eIF2 in an *in vitro* assay for 80S initiation complex formation and synthesis of the first peptide bond. Interestingly, FUN12 is required in yeast for the block to reinitiation at uORF4 in *GCN4* mRNA under conditions where the ability of eIF2 to link initiator $tRNA^{Met}$ to ribosomes is compromised by its phosphorylation. This may indicate that FUN12 competes with eIF2 *in vivo* for limiting amounts of initiator $tRNA^{Met}$, and this is important for reducing the concentration of eIF2/GTP/initiator $tRNA^{Met}$ ternary complexes to the critical level needed to stimulate *GCN4* mRNA translation. There are indications that FUN12 is also required *in vivo* for the cap-independent translation of M or L-A viral mRNAs. cDNAs encoding the human homolog of FUN12 have been isolated, and it was shown that a truncated form of the human protein can complement a *fun12* deletion

in yeast. Thus, the function of FUN12 in providing an alternative mechanism for translation initiation appears to be conserved between fungi and mammals.

LABORATORY OF EUKARYOTIC GENE REGULATION

Chief
Alan G. Hinnebusch, Ph.D.

Tenure-Track Investigators
Thomas Dever, Ph.D.
Henry Levin, Ph.D.

Senior Fellow
Weimin Yang, Ph.D.

Post-Doctoral Fellows
James Anderson, Ph.D.
Katsura Asano, Ph.D.
Sang Ki Choi, Ph.D.
Van Dinh Dang, Ph.D.
Jinsheng Dong, Ph.D.
Minerva Garcia, Ph.D.
Sangtae Han, Ph.D.
Makiko Kobayashi, Ph.D.
Joon Haeng Lee, Ph.D.
Jia-Hwei Lin, Ph.D.
Teresa McDuffie-Singleton, Ph.D.
Richard McVeigh, Ph.D.
Krishnamurthy Natarajan, Ph.D.
Graham Pavitt, Ph.D.
Hongfang Qiu, Ph.D.
Evelyn Sattlegger, Ph.D.

Pre-Doctoral Fellows
Jeannie Kim Choi
Hanse Chung
Lin Phan
Keith Remo
Scott Steele

Guest Researchers
David Balasundaram, Ph.D.
James Bodley, Ph.D.
Patrick Romano, Ph.D.

Senior Technical Specialist
Belinda Jackson

ANNUAL BIBLIOGRAPHY

Asano K, Vornlocher HP, Richter-Cook NJ, Merrick WC, Hinnebusch AG, Hershey JWB. Structure of cDNAs encoding human eIF3 subunits: possible roles in RNA binding and macromolecular assembly. J Biol Chem 1997; 272:27042-52.

Dever TE. Using GCN4 as a reporter of eIF2α phosphorylation and translational regulation in yeast. Methods 1997;11:403-17.

Gale MJ, Korth MJ, Tang NM, Tan S-L, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 1997;230:217-27.

Hinnebusch AG. Translational regulation of yeast GCN4: a window on factors that control initiator-tRNA binding to the ribosome. J Biol Chem 1997;272:21661-4.

Huth JR, Bewley CA, Jackson BM, Hinnebusch AG, Clore, GM, Gronenborn AM. Design of an expression system for detecting folded protein domains and mapping macromolecular interactions by NMR. Protein Science 1997;6:2359-2364.

Kawagishi-Kobayashi M, Silverman JB, Ung TL, Dever TE. Regulation of the protein kinase PKR by the vaccinia virus pseudosubstrate inhibitor K3L is dependent on residues conserved between the K3L protein and the PKR substrate eIF2α. Mol Cell Biol 1997;17:4146-58.

Levin, HL. An unusual mechanism of self-primed reverse transcription requires RNase H for cleavage of an RNA duplex. Mol Cell Biol 1996;16:5645-54.

Levin HL. It's prime time for reverse transcriptase. Cell 1997;88:5-8.

Lin J, Levin HL. A complex structure in the mRNA of Tf1 is recognized and cleaved to generate the primer of reverse transcription. Genes Dev 1997;11:270-85.

Lin J, Levin HL. Self-primed reverse transcription is a mechanism shared by several LTR-containing retrotransposons. RNA 1997;3:952-3.

Marton MJ, Vazquez de Aldana CR, Qiu H, Chakraburtty K, Hinnebusch AG. Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2α kinase GCN2. Mol Cell Biol 1997;17:4474-89.

Pavitt GD, Yang W, Hinnebusch AG. Homologous segments in three subunits of the guanine nucleotide exchange factor eIF2B mediate translational regulation by phosphorylation of eIF2. Mol Cell Biol 1997;17:1298-1313.

Rolfes RJ, Zhang F, Hinnebusch AG. The transcriptional activators BAS1, BAS2, and ABF1 bind positive regulatory sites as the critical elements for adenine regulation of ADE5,7. J Biol Chem 1997;272:13343-54.

Taylor DR, Lee SB, Romano PR, Marshak DR, Hinnebusch AG, Esteban M, Mathews MB. Autophosphorylation sites participate in the activation of the double-stranded-RNA-activated protein kinase PKR. Mol Cell Biol 1996;16:6295-302.

Zhang F, Kirouac M, Zhu N, Hinnebusch AG, Rolfes RJ. Evidence that complex formation by Bas1p and Bas2p (Pho2p) unmasks the activation function of Bas1p in an adenine-repressible step of ADE gene transcription. Mol Cell Biol 1997;17:3272-83.

Zhu S, Romano PR, Wek RC. Ribosome targeting of PKR is mediated by two double-stranded RNA-binding domains and facilitates in vivo phosphorylation of eukaryotic initiation factor-2. J Biol Chem 1997;272:14434-41.

LABORATORY OF INTEGRATIVE AND MEDICAL BIOPHYSICS

Ralph Nossal, Ph.D., Chief

The newly established Laboratory of Integrative and Medical Biophysics (LIMB) carries out cross-disciplinary research in biomedical science on complex problems, whose understanding requires the integration of information and concepts derived from many complementary disciplines. Much of the work utilizes specialized experimental and theoretical techniques commonly associated with physical and engineering science research. Moreover, it frequently requires the development and application of novel methodologies to probe multiple, interacting biological processes over a wide range of time and length scales. Current projects are aimed at elucidating physical principles governing fundamental aspects of cell structure transformations involved in cytoskeletal rearrangement and intracellular trafficking, at understanding the link between the macroscopic mechanical properties of cartilage and its ultrastructure, at measuring water transport in tissues and organs noninvasively, at investigating gene alterations and gene expression in small numbers of targeted cells excised from histology samples, and at developing quantitative *in vivo* optical imaging of tissues. Laboratory staff have unique capabilities: we are famliar with, and ready to develop and use advanced physical methods (e.g., photon and neutron scattering, magnetic resonance and optical imaging); we formulate mathematical models; and we develop biomimetic analogs that exhibit biomedically-relevant phenomena. A principal aim of our work is to devise new research and diagnostic modalities, which at present include optical techniques for detecting targets buried deeply in tissue, diffusion-tensor magnetic resonance imaging (MRI) to characterize tissue microstructure and monitor its changes in disease, and Laser Capture Microdissection for molecular pathology and developmental biology.

CELL BIOPHYSICS

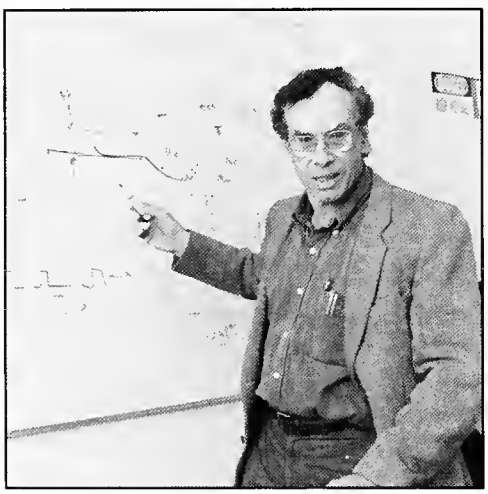
Much of the work of the Section on Cell Biophysics, directed by ***Ralph Nossal***, is aimed at understanding biophysical aspects of cell activities involving the formation of, and structural changes in, supramolecular biological assemblies. Until now, work primarily has involved applications of mathematical and physical theory to data acquired in collaborative studies or from published literature. However, we anticipate an increasing emphasis on self-initiated experimental research as the Laboratory becomes established. Recent projects have addressed questions relating to macromolecular complexes involved in intracellular trafficking, cell locomotion, and structure transformation accompanying cell division and growth.

Physical Analysis of Biological Vesicle Formation. Except for certain small molecules, most materials are taken up into cells by processes that involve vesicularization of the external (plasma) membrane. Also, materials such as nascent polypeptides and recycled cell surface receptors are transported between distinct cellular regions by pathways that seem to involve the formation and dissolution of membrane bound vesicles. Despite the importance of those processes and the vast effort that has led to the identification of numerous relevant molecular factors, little is understood about the physical mechanisms governing the induction and regulation of the membrane transformations involved in endocytosis (i.e., material intake) and intracellular trafficking. In particular, experimental estimates of the bending *moduli* of biological membranes lead one to infer that a very high energy barrier must be overcome if the transformation from an essentially flat plasma membrane to highly curved vesicle (ca. 1000 Å diameter) is to occur. Yet the mechanisms by which this energetically costly transition takes place are poorly understood.

Several events might stimulate vesicle formation. Two such events are of special interest, because there is biological evidence for them: a transient, local variation in membrane charge (due, perhaps, to the phosphorylation of minor phospholipid components or the cytoplasmic tails of embedded receptors); and the formation of vesicle coats from structural proteins that have intrinsic curvature. To investigate these mechanisms, we performed mathematical analysis, based on physical theory, of the imbalance in membrane-bound electrical charge that is required to bend the plasma membrane into a shape commensurate with observed curvatures of endocytotic vesicles. We also examined the energetics of protein coat formation, focusing on the role of the three-armed, multicomponent macromolecular complex, the clathrin triskelion, which is the major coat protein of vesicles formed during receptor-mediated endocytosis.

With respect to the effects of the distribution of bound electronic charge, we found that quite reasonable values of local charge density, perhaps due to phospholipase-D activated PIP_2 production, can introduce the curvature necessary to initiate vesicle formation. However, the resultant charge imbalance can be expected to be transient, and we now are investigating how the kinetics of signal transduction can influence the dynamics of vesicularization. To obtain estimates of the energetics involved in coat formation, we analyzed published data on size distributions of reconstituted clathrin baskets. We developed a minimal statistical thermodynamic theory that allowed us to infer values of bending energies for clathrin arms as well as values of the attractive energies associated with arm interactions. The theory and ancillary data reduction yield quite reasonable values for arm association energies compared with k_BT (which is a measure of the typical kinetic energy of molecules at room temperature), indicating: (1) that relatively simple addition/removal steps can be involved in clathrin coat formation on membrane surfaces (as predicted earlier from topological considerations); (2) why clathrin-coated vesicles fall within a narrow range of sizes. Previous work showed that the energies of bending of a clathrin lattice are similar in magnitude to those associated with bending an equivalently sized membrane patch.

Structural Transitions of Microtubules. The integrity of internal cell structure and overall cell shape depends on many factors. A central role is played by microtubules, which emanate from microtubule organizing centers to impart rigidity and polarity to a cell and its appendages. However, microtubules are labile, undergoing dramatic structural transformations during the cell cycle. Although methods have been worked out for examining the ultrastructure of dried microtubule samples, very few techniques allow study of the dynamical structure of unfixed samples. Hence, we developed new protocols to examine isolated microtubules and microtubule arrays in solution. These involve small angle neutron scattering, which has the advantages that a wide variety of solvent conditions can be investigated and that selected components of macromolecular complexes can be accentuated by varying the D_2O/H_2O content of the scattering system. In collaboration with staff at the Center for Neutron Research at the National Institute of Standards and Technology, we have investigated the structure of microtubules formed under different conditions, including in the presence of taxol and other antimitotic drugs. We have worked out procedures for relating measured scattering cross-sections to the size of the microtubules, as well as to the relative amounts of intact microtubules, partially formed sheet-like or corkscrew structures, and unpolymerized tubulin present in the samples. In accordance with results of electron microscopy studies, we see significant structural differences between, e.g., taxol-associated microtubules and microtubules polymerized in the presence of vinblastin. Moreover, we have been able to study effects not readily observed by other methods, such as those brought about by changing the pH of the solution and by the binding of small oligopeptides to regions of tubulin monomers believed to regulate assembly.


Ralph Nossal

Cell Mechanics. The modification of the actin cytoskeleton is an intrinsic factor in many important cell behaviors. A functional example is the stiffening of cells that occurs when PMN leukocytes are placed in a uniform bath of peptide chemoattractants. Two methods have been used to measure this response: a miniature "cell poker," which involves pushing a small glass bead against a cell surface, and the "cell transit analyzer," which determines the rates at which pliant cells pass through a microporous filter when subjected to hydrostatic pressure. Use of the cell poker is limited because only one cell at a time can be studied and the method cannot be readily adapted to kinetic studies. In contrast, the cell transit analyzer has the advantage that many cells can be examined during a single two- or three-minute period and, because the measurements take only a few milliseconds, cell kinetics can be studied.

Until now the cell transit analyzer has only been employed in a qualitative fashion, because the relationship between macroscopic observations (transit times) and intracellular parameters (viz., the mechanical properties of the cell cytoskeleton) had not been well elucidated. However, by employing a physical-chemical theory of the mechanical behavior of polymer networks, we recently established that cell transit times are quantitatively proportional to the instantaneous shear modulus of the cell cortex, a parameter that can be related to properties of the cortical F-actin matrix. This observation allowed us to analyze published data on ligand-induced changes

of rigidity in PMN leukocytes and rationalize a previously-noted, peculiar, power-law logarithmic dependence of transit time on chemoattractant concentration. We were able to infer a linear-logarithmic relationship between the extent of F-actin polymerization and the logarithm of the chemoattractant concentration. Consequently, comparison with simulations of the dose-response of G-protein activation that is known to occur when chemoattractants bind to receptors on the surfaces of PMN cells suggests a direct linear relationship between ligand binding and actin polymer formation.

Other techniques have been used to study cellular F-actin *in situ*, including light scattering, fluorescent detection of incorporated phalloidin or phallocidin, and electron microscopy. Among these, only light scattering provides a real-time, rapid measure of a change in F-actin content, and none provides functional information relating to the cell activation that occurs, e.g., as a result of ligand binding. We have demonstrated that the cell transit analyzer, in contrast, provides real-time information about cell mechanical response that can be quantitatively linked to structural changes in the cell cytoskeleton. Possible applications include kinetic studies of the signaling pathways, including downstream control loops, which may be involved in receptor-mediated cellular response to peptide chemoattractants. Another use might be to study surface-localized cytoskeletal changes that may be involved in endocytosis and phagocytosis.

TISSUE BIOPHYSICS AND BIOMIMETICS

The Section on Tissue Biophysics and Biomimetics, directed by ***Peter Basser***, carries out research, primarily in tissue biology, directed toward understanding the physical and chemical principles underlying various physiological phenomena. Investigations often involve the development of new physical theories, mathematical and computational models, and biomimetic tissue analogs to aid in the design and interpretation of experiments. The understanding gleaned from these basic studies is often applied to developing new methodologies useful clinically and in biomedical research.

Properties of the Extracellular Matrix. The collagen network plays a critical role in determining functional properties of cartilage and other extracellular matrices in health, disease, or development. However, until now, it has not been possible to measure or even characterize mechanical properties of the collagen network independently within the tissue. We have devised a new methodology to ascertain mechanical properties of the network *per se*, rather than of the tissue matrix as a whole. This new approach entails: (1) modeling the cartilage tissue matrix as consisting of two distinct phases, the collagen network and proteoglycans (PG), rather than as a single "solid-like" phase; and (2) using an osmotic stress titration experiment to determine the equilibrium hydrostatic pressure, P_c, caused by tensile stresses developed within the collagen network, as well as the osmotic pressure exerted by the proteoglycans, P_{PG}, as a function of tissue hydration.


Peter Basser

In a pilot study, we used this approach to determine P_c as a function of hydration in several normal human cartilage samples, both native and trypsin-treated, and in cartilage from osteoarthritic (OA) joints. These curves coincide in both normal and trypsin-treated specimens, showing a steep increase in P_c with increasing hydration. In OA specimens, however, the curves are shallower, indicating that the collagen network has become more flaccid. Our findings highlight the role of the stiffness of the collagen network in limiting cartilage hydration, and ensuring a high PG concentration in the matrix. The latter is essential for effective load bearing but is lost in OA. These data also suggest that the loss of stiffness of the collagen network, and not the loss of proteoglycans, may be the incipient event leading to the disintegration of cartilage in OA.

Diffusion Tensor Spectroscopy and Imaging. Diffusion is often the dominant molecular transport mechanism of water and solutes in cells and tissues, and is thus implicated in all physiological processes. Nevertheless, until the advent of diffusion magnetic resonance imaging (D-MRI) methods, one lacked the means to measure water or solute diffusion *in vivo*, non-invasively. Conventional D-MRI is a method to measure a scalar diffusion

constant of water (as well as of some ions and molecules) within a voxel (i.e., a three-dimensional pixel). However, this parameter does not provide enough information with which to characterize diffusion in most biological tissues, particularly in fibrous tissues such as muscle, tendon, ligament, intravertebral disc, kidney medulla, and brain white matter. For these tissues one must describe solvent and solute diffusion using a tensor rather than a scalar diffusivity.

Recently, we developed a new MR imaging modality, Diffusion Tensor MRI (DT-MRI), to measure this diffusion tensor, non-invasively, *in vivo*. New information we are able to obtain from this method includes images of diffusion ellipsoids that depict local fiber-tract orientation in tissues, as well as the mean diffusion distances in each direction. Scalar invariants of $\underline{D}$ reveal microstructural details about tissues independent of the laboratory coordinate system. In analogy with quantitative histological or physiological stains, these MR quantities can be used as "stains" for the bulk or average diffusivity, diffusion anisotropy, structural similarity, and degree of fiber-tract coherence or organization.

We are continuing to apply DT-MRI to problems in basic research and in clinical medicine. Collaborations with investigators in NINDS and NIMH have helped advance DT-MRI from "bench to bedside". Specifically, new echo-planar imaging (EPI) sequences permit DW-MRIs to be acquired rapidly enough for a clinical assessment. Interleaving these sequences has increased the spatial resolution of the resulting images; navigator-echo-correction schemes have allowed motion artifacts to be greatly reduced; and eddy-current compensation strategies have reduced image distortion artifacts significantly. Finally, simplifying the experimental design of these MRI experiments and improving our image analysis methods has facilitated the implementation of DT-MRI. Collectively, these developments have encouraged the wide-spread use of this new methodology.

Clinical findings, in studies performed under NINDS protocols with normal volunteers, show that this new imaging modality can be used to improve our ability to segment brain tissue into white matter, gray matter, and CSF compartments. Moreover, diffusion anisotropy in white matter is found to be highly variable in human brain, and much larger than was reported previously in regions such as the *corpus callosum*, pyramidal tracts and optic tracts. In chronic stroke patients, DT-MRI was found to be effective in identifying Wallerian degeneration as well as organized gliosis. In acute stroke, the Trace of the diffusion tensor, which is proportional to the bulk or orientationally averaged diffusivity, has been the most successful imaging parameter proposed to date to visualize a stroke in progress. Preliminary studies have also shown DT-MRI to be useful in assessing postnatal changes in the cortex of living cat brain. Presently, we are developing new MRI "stains" for use in assessing the degree of tissue organization, fiber tract continuity, and fiber tract connectivity.

MEDICAL BIOPHYSICS

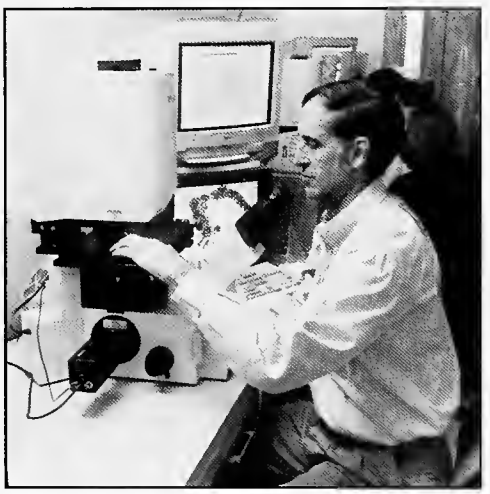
The Section on Medical Biophysics, headed by ***Robert Bonner***, develops new biophysical and optical methodologies for biomedical research and clinical applications. Current new technologies being developed include: (1) Laser Capture Microdissection and molecular diagnosis; (2) optical coherence microscopy of tissues at depths heretofore inaccessible to optical imaging for noninvasive study, e.g., of developing embryos and transplant organs; (3) methods for quantitation of *in vivo* photochemistry (e.g., formation of cataracts and macular degeneration of aging); and (4) minimally invasive, 3D quantitative clinical optical imaging of the concentration of specific fluorescent macromolecular labels.

Laser Capture Microdissection. This program of biophysical technology development is a complex multistage endeavor that has led to the invention of laser capture microdissection (LCM) and to plans for future applications of this evolving method to a variety of critical scientific and clinical problems. In the last year, the original concept for LCM has been taken through a variety of feasibility tests, a series of technical refinements, and finally the establishment of a CRADA for commercial development.

LCM uses a high-resolution laser-based cell transfer technique within a microscope. It entails placing a thin transparent thermoplastic film on a supporting substrate onto a tissue section, visualizing the tissue microscopically, and selectively transferring the cells of interest to the film by a short, focused laser pulse. The tissue transferred to the film retains its original morphology, thereby allowing microscopic verification of the transfer efficacy. Transfers can be done in substantially less time than by manual microdissection. The film, with the attached tissue, is removed from the section and placed into a DNA-, an RNA-, or a protein-extraction buffer

prior to molecular analysis. The use of sterile, disposable transfer films minimizes potential contamination, which is particularly important for PCR-based analyses.

This activity has involved multidisciplinary applied physical science and strong communication with the intramural molecular diagnostic community. Once a robust routine technology base is established (including targeted single cell transfer, specialized integrated LCM microscopes, and appropriate biophysical and biochemical procedures), our focus will turn to critical scientific problems such as using the technology for quantitative determination of differential gene expression in development, in natural organ function, and in pathology. A major, immediate effort (within an interdisciplinary group including LP/NCI, BEIP, DCRT, CRADA partners, and extramural multicenter institutions using LCM) will be to use LCM as a tool for clinical research into the relation between the human genome and anatomy, including the differential expression of genes in normal and pathological cells selected from histopathology specimens. In addition to use in understanding disease processes at the molecular level of DNA alterations (i.e., in infectious disease and cancer), LCM can be used in studies of the quantitative differential expression of genes in developing systems and in natural response of cells to external stimuli. We also will seek to develop optimized specific clinical diagnostic techniques (e.g., rapid determination of drug resistant TB and of molecular staging of precancers and cancers) that make use of this new technology.


Robert Bonner

LCM training workshops have been organized for investigators from around the world. We also are establishing an Internet-based microdissection training and feedback network (MTFN) to disseminate new methodologies in molecular pathology to a growing community of interested investigators.

Optical Coherence Microscopy. Optical-coherence reflectometry (OCDR) was first introduced as a method for the high-resolution probing of reflections from components of fiber-optic systems. We have examined the applications of this technique to imaging and characterization of biological tissues. Using prototype instruments built in our laboratory, we have succeeded in imaging subsurface structures in living skin and other tissues. To help understand the limits of contrast and resolution that can be attained with this new type of microscopy, mathematical models of the propagation of partially coherent light in dense tissue have been constructed and tested in experiments on tissues and tissue phantoms. Our results show that, without staining or modifying a tissue in any way, structures embedded one to two millimeters within skin tissue can be imaged with a resolution of about 10 μm. We continue to interact with the industry developing refined optical coherence imaging systems, carrying out feasibility tests in such clinically important tasks as noninvasive and sterile assessment of transplant organs, imaging of pathological lesions near tissue surfaces, and in research applications requiring noninvasive imaging of developing bone, small embryos, and organ culture systems.

TISSUE CHARACTERIZATION BY OPTICAL MEANS

Many of the research projects carried out by members of the Laboratory involve the interaction of light with tissue. Examples are low-coherence interferometric microscopy, diffusive wave and time-gated optical imaging, laser microsurgery, laser Doppler blood-flow measurements, photodynamic therapy of cancer, and noninvasive platelet assessment. In order to make these techniques more quantitative and, also, to develop new noninvasive techniques for tissue spectroscopy, we have undertaken physical and mathematical modeling of light propagation in biological tissues and turbid media. Members of the Laboratory have worked together in teams whose composition varies from project to project.

Mathematical Analysis. Non-ionizing visible and near-infrared light may be used to characterize tissue structures noninvasively by a combination of its wavelength-dependent differential scattering and absorption. One potential application is to detect abnormal regions (e.g., tumors) embedded deeply within otherwise normal tissue. However, since tissue strongly scatters light, conventional optical imaging is of limited practicality when examining structures lying more than a few hundred microns below an illuminated surface. In principle,

improvement of spatial resolution and contrast can be obtained by using time-gated detection of photons to select those photons which move on relatively short paths while traversing the tissue. However, the interpretation of measured signals is complicated by the diffuse nature of the re-emitted light and the sampling biases of different regions of complex tissue. Hence, there is a continuing need to develop mathematical theory to advance the uses of light in medical diagnosis.

Previously we devised analytical equations characterizing various parameters of photons re-emitted from an illuminated tissue surface. These expressions have been used to interpret empirical observations on living tissues and to quantify a variety of clinical measurements. During the past year we derived and began the evaluation of an inverse algorithm to infer the optical properties of abnormal tissue embedded in an optically turbid background which, to a first approximation, can be taken as optically homogeneous. It is of great significance that we are able to account for the fact that the optical properties of the abnormal region often differ only slightly from those of the surrounding normal tissue. The derived algorithm is based on a photon random walk model that uses different time-dependent point spread functions to calculate the diffusive and absorptive contrasts obtained from time-of-flight measurements. Because the diffusive and absorptive contrast functions have differing time-dependent behaviors, one can discriminate between absorbing and scattering contributions to the total detected contrast. In addition to providing information about the optical coefficients and location of the unusual region, calculations based on the algorithm also yield a measure of the size of the inclusion. Working in collaboration with investigators from the Department of Medical Physics of University College, London, we have examined time-of-flight measurements performed on tissue-like phantoms that contain a single aberrant mass and have found that our method is able to determine optical parameters to an accuracy of 10-15%, even for a target as small as 5 mm hidden in a 55 mm thick slab having scattering characteristics similar to those of breast tissue. Although in its present version the algorithm cannot reconstruct the detailed shape of the target, we obtained equivalent volumes whose average dimensions differ from the known dimensions of the targets by less than 20%. This study presently is being extended to include multiple targets and boundary and shape effects.

New Techniques. Such improved understanding of the physics of photon migration in tissues will also form the basis for new simple noninvasive technologies for monitoring and screening important physiological variables such as blood and tissue oxygenation, hematocrit, and bilirubin concentration. Recently, working in collaboration with the Laboratory of Cardiac Energetics, NHLBI, we applied similar approaches to remote detection of tissue spectra from which myoglobin oxygenation and cytochrome C redox state can be determined near the epicardial surface of a beating heart *in situ*. However the prospects for noninvasive 3-D localization and detection of specific pathologies are inherently limited due to weak absorption signals of relevant biological molecules (e.g., glucose absorption in diabetics), poor specificity of strong absorption signals due to hemoglobin, and strong scattering centers (e.g., due to neovascularization and microcalcifications in breast cancer). To overcome these limitations we have recently concentrated on imaging specific populations of fluorescently labeled cells. Studies have been carried out to develop an image reconstruction algorithm to quantify concentrations of fluorophores located at discrete sites below the surface of an optically turbid medium. These are being used in a collaborative project aimed at developing a non-destructive optical biopsy technique for the quantification of lymphocyte infiltration of minor salivary glands in the autoimmune disease known as Sjögren's Syndrome. This project may develop a paradigm for optical detection that addresses a critical aspect of diffuse imaging of tissue, namely, the specificity of the optical signal of the target relative to the background. To further such quantitative, *in vivo*, clinical fluorescence imaging techniques, we have developed a theoretical framework that describes photon path dispersion within the tissue and provides a robust image reconstruction algorithm to determine the fluorophore concentration at a given depth. The algorithm has been successfully tested on a tissue-like phantom, and we now are building a prototype clinical biopsy system in cooperation with clinicians in the National Institute of Dental Research.

LABORATORY OF INTEGRATIVE AND MEDICAL BIOPHYSICS

Chief
Ralph Nossal, Ph.D.

Principal Investigators
Peter Basser, Ph.D.
Robert F. Bonner, Ph.D.

Senior Fellow
Amir Gandjbakhche, Ph.D.

Guest Researchers
Victor Chernomordik, Ph.D.

Muragappan Muthukumar, Ph.D.
Vinay Prabhu, Ph.D.

ANNUAL BIBLIOGRAPHY

Agah R, Gandjbakhche AH, Motamedi M, Nossal R, Bonner RF. Dynamics of temperature dependent optical properties of tissue: dependence on thermally induced alteration. IEEE Trans Biomed Eng 1996;43:839-46.

Basser PJ. New histological and physiological stains derived from diffusion tensor MR images. Ann N Y Acad Sci 1997;820:123-38.

Basser PJ, Mattiello J, LeBihan D. Method and System for Measuring the Diffusion Tensor and for Diffusion Tensor Imaging. US Patent#5,539,310, July 23, 1996.

Basser PJ, Pierpaoli C. Microstructural and physiological features of tissues elucidated by quantitative-diffusion-tensor MRI. J Magn Reson B 1996;111:209-19.

Bonner RF, Emmert-Buck M, Cole K, Pohida T, Chuaqui R, Goldstein S, Liotta LA. Laser capture microdissection: modern analysis of tissue. Science 1997;278:1481-3.

Chernomordik V, Nossal R, Gandjbakhche AH. Point spread functions of photons in time-resolved transillumination experiments using simple scaling arguments. Phys Med Biol 1996;23:1857-61.

Dean-Clower E, Vortmeyer AO, Bonner RF, Emmert-Buck M, Zhuang Z, Liotta LA. Microdissection-based genetic discovery and analysis applied to cancer progression. Cancer J Sci Am 1997;3:259-65.

Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA. Laser capture microdissection. Science 1996;274:998-1001.

Gandjbakhche AH, Bonner RF, Nossal R, Weiss GH. Effects of multiple passage probability on fluorescent signals from biological media. Appl Opt 1997;36:4613-9.

Gandjbakhche AH, Chernomordik V, Bonner RF,Hebden JC, Nossal R. Use of time-dependent contrast functions to discriminate between the scattering and absorption properties of abnormal regions hidden within a tissue-like model. In: Alfano RR, Chance B, eds. Optical tomography, photon migration, and spectroscopy of tissue and model media II. Proc SPIE 1997;2979:211-7.

Gandjbakhche AH, Chernomordik V, Bonner RF, Nossal R, Weiss GH, Hebden J. Optical detection of abnormally absorbing regions in tissue. In: RR Alfano, JG Fujimoto, eds. OSA Trends in Optics and Photonics on Advances in Optical Imaging and Photon Migration. (Optical Society of America, Washington DC 1996), Vol 2. pp183-9.

Gandjbakhche AH, Gannot I. Quantitative fluorescence imaging of specific markers of diseased tissue. IEEE Sel Topics in Quant Elec 1996;2:914-21.

Gannot I, Bonner RF, Gannot G, Fox P, Yoo J, Waynant R, Gandjbakhche AH. Quantitative detection of multiple flourophore sites as a tool for diagnosis and monitoring disease progression in salivary glands. In: Alfano RR, Chance B, eds. Optical Tomography, photon migration, and spectroscopy of tissue and model media II. Proc SPIE 1997;2979:151-6.

Mattiello J, Basser PJ, LeBihan D. The b matrix in diffusion tensor echo-planar imaging. Magn Reson Med 1997;37:292-300.

Nossal R. Mechanical properties of biological gels. Physica A 1996;231:265-76.

Pierpaoli C, Basser PJ. Toward a quantitative assessment of diffusion anisotropy. Magn Reson Med 1996;36:893-906.

Pierpaoli C, Jezzard P, Basser PJ, Barnett A, Di Chiro G. Diffusion tensor MR imaging of the human brain. Radiology 1996;201:637-48.

LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

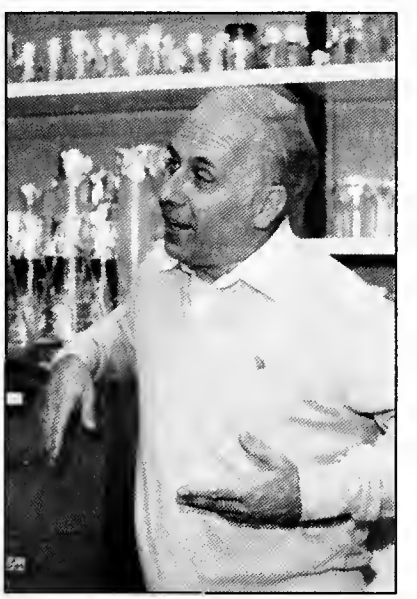
Heiner Westphal, M.D., Chief

The Laboratory generates gene-altered mice to study the molecular genetics of genes that control pattern formation in the embryo, T cell development, and genomic imprinting. These mutant mice also serve as experimental paradigms of human diseases.

MOLECULAR GENETICS OF MOUSE DEVELOPMENT

Genes that regulate vertebrate development or that are affected in human hereditary disorders are the topic of current and projected research in the Section on Transgene Regulation, directed by ***Heiner Westphal***. His studies of the mouse embryo address a critical phase in development when precursor cells receive signals that channel them toward regional specificity and differentiation. *Sonic Hedgehog* knockout mutants, the topic of one of the projects, exemplify the profound consequences of a disruption in a chain of commands that sets off this process. Likewise, the *Lhx3/4* double knockout mice are proof of the fundamental importance of transcriptional regulators in mediating the change from a committed to a specialized cell. The projects illuminate the actions of genes that are of fundamental importance for the development and organization of functional tissue systems. At the same time, the group has generated animal models of tumor suppression (see 1996 Annual Report) and of Hirschsprung's disease, a disorder characterized by neuronal loss in the gastrointestinal tract.

***Sonic Hedgehog* (*Shh*)**. This gene plays important roles in ventral patterning along the anterior-posterior axis of the developing central nervous system and in influencing pattern of cell types elsewhere in the developing vertebrate embryo. In collaboration with the laboratory of Philip Beachy at Johns Hopkins University, we have analyzed the consequences of loss of *Shh* function in the mouse. More recently, we have become interested in *Shh* function during limb development. Observations of the knockout embryo have established that the gene is essential for the outgrowth of distal limb structures. *Shh* is also required for anterior-posterior patterning of the limbs since grafts of *Shh* mutant mesenchyme are unable to induce patterning activity. *Shh* controls limb development by mediating the action of specific growth factors and transcription factors.



Heiner Westphal

LIM-Homeobox (*Lhx*) Genes. This family of developmental transcription factors regulates important steps of organogenesis. *Lhx2*, *Lhx5*, and *Lhx7*, closely related members of this gene family, are essential for pattern formation, particularly in the developing central nervous system (CNS). Knockout analyses of these genes are in progress to address their combinatorial functions in the CNS and elsewhere in the developing embryo. *Lhx3* and *Lhx4*, another closely related pair of genes, are responsible for the formation of the adenohypophysis, and also play an essential role in motoneuron innervation along the spinal cord, a function currently being assessed via double loss-of-function analysis. The onset of *Lhx*3 and *Lhx*4 expression coincides with the formation of Rathke's pouch, the primordium of the pituitary gland. Our studies of mouse embryos that lack the function of *Lhx*3, *Lhx*4, or both, have shown that the organ forms in a stepwise fashion. Both genes act during the formation of a definitive pouch, each being able to substitute for the other. Thereafter, *Lhx*3 controls a critical step of pituitary fate commitment. Later, *Lhx*3 as well as *Lhx*4 regulate the proliferation and differentiation of pituitary-specific cell lineages. Thus, *Lhx*3 and *Lhx*4 dictate pituitary organ identity by controlling developmental decisions at multiple stages of organogenesis.

Glial Cell Line-Derived Neurotrophic Factor. Glial cell line-derived neurotrophic factor (GDNF) was isolated as a neurotrophic factor for midbrain dopaminergic neurons. In view of its neurotrophic activity on a wide range

of neuronal populations *in vitro* and *in vivo*, GDNF is being considered as a potential therapeutic agent for neuronal disorders. During mammalian development, GDNF is expressed not only in the nervous system, but also very prominently in the metanephric kidney and in the gastrointestinal tract, suggesting possible functions during organogenesis. We have investigated the role of GDNF during development by generating a null mutation in the murine GDNF locus. Aided by research groups in Denver and in Helsinki, we were able to demonstrate that mutant mice show kidney agenesis or dysgenesis and defective enteric innervation. GDNF induces ureter bud formation and branching during metanephros development. In addition, the factor is essential for proper innervation of the gastrointestinal tract. We are presently examining the role of GDNF in the development of the enteric nervous system and, in this context, attempting to rescue enteric innervation in embryonic gut explants that lack GDNF function. A role of the GDNF *locus* in Hirschsprung's disease is being assessed as well. It appears that partial loss of enteric neurons is sufficient to cause dysfunction of gut peristalsis and even death among the population of GDNF mutant mice.

COLLABORATIVE STUDIES

The Embryo Microinjection Facility, an integral part of the Section, is a barrier mouse facility that houses up to 3,000 mice and contains a centrally located animal manipulation and embryo microinjection laboratory, in which gene-altered mice are generated. Groups outside this laboratory often request assistance in designing their own experiments in this regard. As a result, the Section on Transgene Regulation becomes acquainted with new and important research avenues, especially those located at the interface between molecular genetics and medicine. This has had a very positive influence on our own research concepts and has helped us to attract a number of excellent coworkers, who have been trained both as physicians and as molecular biologists.

Uteroglobin. In a joint project with Anil Mukherjee and his colleagues at NICHD, we have studied the function of uteroglobin, using a loss-of-function approach. In mice lacking uteroglobin gene function, severe renal disease develops that is associated with massive glomerular deposition of predominantly multimeric fibronectin. Uteroglobin binds to fibronectin with high affinity. This binding appears to be required to counteract fibronectin self-aggregation and deposition. Uteroglobin is thus essential for maintaining normal renal function in mice. Uteroglobin gene defects may well underlie human glomerular disease that is accompanied by fibronectin deposits. Details may be found elsewhere in this volume (p 90).

Nhlh2. A second project, carried out with Ilan Kirsch and his colleagues at NCI, addressed the function of the transcription factor Nhlh2. Loss of this gene results in disruption of the hypothalamic-pituitary axis in mice. Male knockout mice are hypogonadal and infertile. The same holds for females unless housed together with males, suggesting that male pheromones may be able to compensate for their genetic defect. Both males and females exhibit progressive adult-onset obesity. These results support a role for Nhlh2 in the onset of puberty and in the regulation of body weight metabolism. The gene is a candidate for human disorders in which both are compromised.

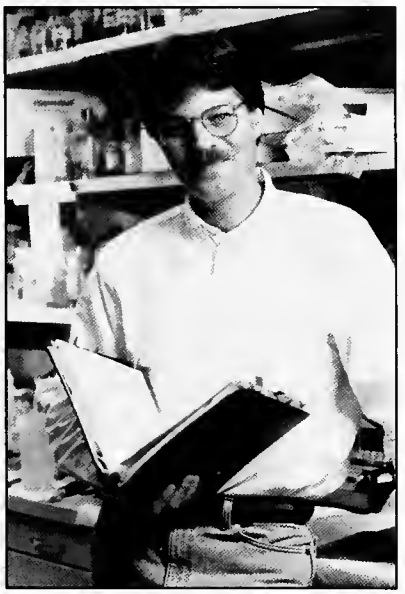
T CELL DEVELOPMENT

Research in the Unit on Cellular and Developmental Biology, led by ***Paul Love***, is directed at understanding the cellular and genetic events that control normal T cell development. Current studies focus on the role in thymocyte maturation of signals transduced by cell surface receptors, particularly the T cell antigen receptor (TCR). In mature T cells, the TCR complex is centrally involved in antigen recognition, T cell activation, and in cell-mediated immunity. In developing thymocytes, TCR signals are important for maturation and thymic (positive and negative) selection. How the TCR is capable of controlling these various processes remains a central question in immunology and is relevant to understanding human immunodeficiency and autoimmune disorders.

Role of TCR Signaling Molecules in T Cell Development. Our main focus of current research is the elucidation of the role of T cell antigen receptor (TCR) signal transduction in thymocyte development. Our recent work has been aimed at systematically dissecting and characterizing the function of the multiple signal transducing sequences contained within the TCR. TCR signal transduction sequences (termed Immunoreceptor Tyrosine-based Activation Motifs; ITAMs) are contained within four distinct subunits of the multimeric TCR complex (ζ, CD3-γ, CD3-δ, CD3-ε) and are triplicated in the ζ chain cytoplasmic domain. Di-tyrosine residues within ITAMs

are phosphorylated upon TCR engagement, and their function is to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell activation cascade. Although conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses that are controlled by the TCR may be regulated, in part, by distinct ITAMs.

Experiments performed in this laboratory have included overexpression of various TCR signaling subunits in transgenic mice with the expectation that perturbations in T cell development, if observed, would provide insight into the function of these molecules. The results of these studies suggest that individual TCR subunits perform specialized functions at different points in development, as components of both the Pre-TCR (a signaling complex expressed in early thymocytes that includes some but not all of the TCR subunits) and the TCR. In addition, these experiments indicate that regulated expression of TCR signaling subunits during development is essential for ensuring that the appropriate signals are delivered to thymocytes at specific stages of maturation.


Paul Love

To examine the developmental role of ζ chain, the major TCR signaling subunit, ζ-deficient ($\zeta^{-/-}$) mice were generated by gene targeting. The phenotype of these mice demonstrated a critical function for ζ chain in promoting TCR surface expression and T cell development. The importance of ζ-chain ITAM signals was then examined by reconstituting TCR surface expression in $\zeta^{-/-}$ mice using transgenes that encode full-length ζ-chain or ζ-chain variants lacking one, two, or all three ITAMs. Examination of T cell development in these mice led to the striking finding that ζ-chain signals are not specifically required for T cell maturation, provided that TCR surface expression is restored and the remaining (CD3) signaling chains are present in the TCR complex. Our further investigation of these mice has shown that the ζ-ITAMs are required for efficient thymocyte positive selection (a developmental process that ensures maturation of self-educated T cells and prevents maturation of potentially auto-reactive T cells). These results identify a previously unrealized function for the multiple TCR-ITAMs in amplification of TCR signals during thymocyte selection. Similar approaches are currently being used to examine the role of other TCR signal transducing subunits (e.g., CD3ε) in T cell development and selection.

ζ belongs to a family of proteins [ζ , η (an alternatively spliced form of ζ), and FcεR1γ] that are structurally and functionally related. Interestingly, although the majority of T cells express TCRs that include ζζ homodimers, specific subsets of mature T cells express TCRs that include heterodimers of ζ and FcεR1γ. By mating $\zeta(\eta)^{-/-}$ mice with FcεR1γ $^{-/-}$ ($\gamma^{-/-}$) mice, we have recently generated mice that lack expression of all ζ-family proteins. The $\zeta/\eta^{-/-}$ x $\gamma^{-/-}$ mice have proved especially useful in establishing the role of individual ζ-family proteins in T cell development.

Role of CD5 in T Cell Development and Selection. Accumulating data indicate that another cell surface receptor, CD5, plays an important role in thymocyte selection by influencing signaling through the TCR. CD5 surface expression is regulated by TCR engagement and varies between individual thymocytes depending upon the affinity of their TCR for positively selecting ligands.

To determine if the level of CD5 expression influences the outcome of thymocyte selection, we generated transgenic mice that overexpress CD5 throughout development. Overexpression of CD5 was found to significantly impair positive selection of some thymocytes (those that normally express low levels of CD5) but not of others (those that normally express high levels of CD5). Collectively, these findings support a role for CD5-mediated signals in (negatively) modulating TCR signal transduction, thereby influencing the outcome of thymocyte selection. The ability of thymocytes to regulate their surface expression of CD5 suggests that there are mechanisms by which thymocytes can "fine tune" the TCR signaling response. This potential for signal modulation may be particularly useful in generating the maximum possible TCR diversity in the mature T cell repertoire. A probable mechanism for this function is via the activation-induced binding of regulatory molecule(s) to ITAM-like tyrosine containing sequences within the CD5 cytoplasmic tail.

To directly assess the importance of the CD5-cytoplasmic domain, transgenic mice that express a tailless form of CD5 (mCD5) were generated. Both the intact and mCD5 transgenes were then used to reconstitute CD5

surface expression in $CD5^{-/-}$ mice. These experiments revealed a critical function for the cytoplasmic domain in CD5 signaling. We are currently attempting to identify molecules that interact with CD5 and which may be involved in regulating signal transduction by the TCR.

Identification of Novel Genes Involved in T Cell Development. We have recently initiated a search for novel genes that may be important for thymocyte maturation. Using a reverse-transcriptase PCR (RT-PCR)-based strategy that employs degenerate oligonucleotides specific for certain classes of proteins (including protein tyrosine kinases, protein tyrosine phosphatases and homeobox genes), RNA from early fetal (day 13) or adult thymus is being screened. Using this approach, we have identified a novel nonreceptor protein tyrosine kinase gene expressed in early thymocytes (*txk*). Sequence analysis indicates that Txk belongs to the Tec family of protein tyrosine kinases (PTK). Txk is expressed in thymocytes as early as fetal day 13.5, and its expression continues throughout development. *txk* transcripts are present in peripheral T cells and in mast cell lines, but are not detectable in B cell, macrophage /monocyte cell lines or in non-hematopoietic fetal or in adult tissues. In both thymocytes and T cells, *txk* transcripts are downregulated after activation by TCR cross-linking. Transgenic mice that overexpress wild-type and dominant negative (kinase dead) forms of Txk have been generated to determine the possible role of this PTK in T cell development. The results of these studies indicate that Txk is involved in regulating thymocyte positive selection and mature T cell activation.

We have also utilized RT-PCR to identify potentially novel homeobox-containing genes in the early fetal thymus. Homeobox genes are members of a large family of genes that encode putative transcription factors functioning in development. Each of these genes contains a conserved sequence (homeobox) that encodes a region of the protein (homeodomain) thought to mediate DNA binding. Homeodomain proteins are known to play a role in determining cell fate along the anterior-posterior body axis and in various organs; however, their expression in the developing thymus has not been exhaustively explored. Once identified, our expertise in genetic manipulation will be used to generate transgenic and knockout (null) mutations of these genes.

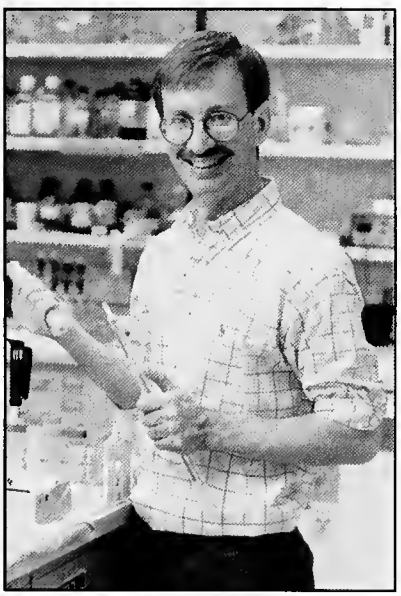
GENOMIC IMPRINTING

Research in the Unit on Genomic Imprinting, which is led by ***Karl Pfeifer***, is directed at understanding the molecular basis for an unusual form of gene regulation, in which expression of certain genes is restricted to either the maternal or to the paternal allele. Genomic imprinting has significant ramifications in developmental biology, and disruptions in allele-specific expression are implicated in several developmental disorders and in a wide variety of tumors. In addition, examination of the regulatory mechanisms underlying the allele-restricted expression offers an exceptional model system in which to investigate the role of chromosome structure in regulating gene expression. In a single nucleus, one allele is expressed while the second allele, of identical nucleotide sequence, is silent. This phenomenon thus implicates a distinct chromosome organization or structure as being responsible for the differential expression.

We are focusing on mouse distal chromosome 7, a region that contains at least five imprinted genes. H19, p57Kip2, and Mash-2 are expressed from the maternally inherited chromosome, while Ins-2 and Igf-2 are expressed from the paternal chromosome. This region is syntenic with human chromosome 11p15.5, where disruption of normal imprinted regulation is associated with Beckwith-Wiedemann Syndrome and with several childhood tumors. Recently, KVLQT1, a gene encoding a potassium channel has been mapped to 11p15.5. Mutations in KVLQT1 increase susceptibility to long QT syndrome.

Maternal Specific Expression of *H19*. The *H19* gene is highly expressed during mouse development in a tissue-specific and stage-dependent manner. We have developed a transgene system to examine the genetic mechanisms for developmentally regulated and allele-specific expression of *H19*. The results have identified 5′ promoter elements and 3′ enhancers required for tissue-specific expression of *H19*. In addition, these results identify two regions required for maternal-specific expression. One primary region is upstream of the *H19* promoter and includes nucleotide sequences shown to be differentially methylated in male and female gametes. A second region lies within the *H19* coding sequences. Expression of *H19* transgenes is completely independent of position and copy number. However, imprinting of these transgenes is copy number-dependent, suggesting that all elements essential for normal imprinting of *H19* have not yet been isolated. Therefore, we have generated additional transgenic lines using Bacterial Artificial Chromosome (BAC) clones, and are currently analyzing these mice.

Coordinate Expression of *H19* and *Igf-2*. *H19* and *Igf-2* are approximately 90 kb apart on chromosome 7 and are transcribed from the same DNA strand. The two genes share upstream enhancer elements, and thus their patterns of expression are strikingly similar throughout mouse development. However, *Igf-2* is expressed only from the paternal chromosome. Disruptions of the *H19* gene and upstream regulatory sequences results in a biallelic pattern of expression of *Igf-2*. To understand the mechanistic significance of the opposite imprinting of these genes, we have generated targeted mutations specific to the *H19* imprinting control elements, which were identified using transgenic mice. These mutations keep the *H19* promoter and coding sequences intact. We will examine allele-specific expression of *Igf-2* to distinguish between two models for coordinated expression/imprinting of the cluster. The first model suggests that the two genes compete for a common set of enhancers and predicts that the genes can never be expressed from the same chromosome. The second model suggests that a chromosome-structure-regulated boundary domain prevents utilization of the shared enhancer elements in a chromosome-specific manner.


Karl Pfeifer

Identification of the Mouse *Kvlqt*1 Gene. We are interested in identifying novel genes in the mouse distal 7 cluster. To this end, we have generated a physical map of the region and have identified BAC and P1 clones that span the entire region from upstream of *p57Kip2* to downstream of *H19*. These clones have been used as probes to identify novel transcripts. One transcript is the mouse homolog of *KVLQT*1, recently identified by positional cloning methods to find genes associated with long QT syndrome. Human *KVLQT*1 is expressed form the maternal chromosome, at least in fetal tissues. We have determined the expression patterns of mouse *Kvlqt*1. The gene is highly expressed in kidney, lung, placenta, uterus, and gut, as well as in heart. While initially expressed form the maternal chromosome, the paternal mouse *Kvlqt*1 gradually becomes activated during embryogenesis. By day 14 (postnatal), expression is biallelic. These results, if applicable to humans, explain the lack of bias in parental origin noted for long QT syndrome.

LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

Chief
Heiner Westphal, M.D.

Tenure-Track Investigators
Paul Love, M.D., Ph.D.
Karl Pfeifer, Ph.D

Senior Fellows
Hui Sheng, M.D., Ph.D.

Staff Veterinarians
Alexander Grinberg, D.V.M.
Eric Lee, D.V.M.

Post-Doctoral Fellows
Alan Agulnick, Ph.D.
Hala Azzam, Ph.D.
Yi-Jun Guo, M.D., Ph.D.
Ying Litingtung, Ph.D.
Liya Shen, Ph.D.
Connie Sommers, Ph.D.
Svetlana Shtrom, M.D.
Madulika Srivastava, Ph.D.
Naoyuki Takuma, M.D.
Tsuyoshi Yamashita, M.D., Ph.D.
Yangu Zhao, Ph.D.

Pre-Doctoral Fellows
Ahmed Amini
Susan Baldwin
Debasis Chakrabarti
Stephen Eacker
Todd Gould
Sandra Hsieh
Elizabeth Ives
Kin Lui
Howard Shen
Andreas Tomac
Chin-Hong Tsay

Guest Researchers
Ohad Birk, M.D., Ph.D.
Chin Chiang, Ph.D.
James Kong
James Vary
Kenneth Woodside

Senior Technical Specialist
Sing-Ping Huang

ANNUAL BIBLIOGRAPHY

Agulnick AD, Taira, M, Breen JJ, Tanaka T, Dawid IB, Westphal H. Interactions of the novel LIM domain binding factor Ldb1 with LIM homeodomain proteins. Nature 1996;384:270-2.

Chiang C, Litingtung Y, Lee EJ, Young K, Corden JL, Westphal H, Beachy P. Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. Nature 1996;383:407-13.

Drago J, Gerfen CR, Westphal H, Steiner, H. Differential regulation of substance P and immediate-early gene expression by cocaine in forebrain of D1 dopamine receptor-deficient mice. Neuroscience 1996;74:813-23.

Franzoso G, Carlson L, Kerstein T, Shores E, Epstein S, Grinberg A, Bennick J, Tran T, Shacter E, Leonardi A, Anver M, Love P, Sher A, Siebenlist U. Critical roles for the Bcl-3 oncoprotein in T cell-mediated immunity, splenic microarchitecture, and germinal center reactions. Immunity 1997;6:479-90.

Gao JL, Wynn TA, Chang Y, Lee EJ, Tiffany HL, Westphal H, Kwon-Chung J, Murphy PM. Impaired host defense, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. J Exp Med 1997;185:1959-68.

Gnarra JR, Ward JM, Porter FD, Wagner JR, Devor DE, Grinberg A, Emmert-Buck MR, Westphal H, Klausner RD, Linehan WM. Defective placental vasculogenesis causes embryonic lethality in VHL knockout mice. Proc Natl Acad Sci USA 1997;94:9102-7.

Good DJ, Porter FD, Mahon KA, Parlow AF, Westphal H, Kirsch IR. Hypogonadism and obesity in mice with a targeted deletion of the Nhlh2 gene. Nat Genet 1997;15:397-401.

Granholm AC, Srivastava N, Mott LJ, Henry S, Henry M, Westphal H, Pichel JG, Shen L, Hoffer BJ. Morphological alterations in the peripheral and central nervous systems of mice lacking GDNF: Immuno-histochemical studies. J Neurosci 1997;17:1168-78.

Liu Y, Grinberg A, Westphal H, McDonald MP, Miller KM, Crawley JN, Hoffmann A, Sandhoff K, Suzuki K, Proia RL. Mouse model of GM2 activator deficiency manifests cerebellar pathology and motor impairment. Proc Natl Acad Sci USA 1997;94:8138-43.

Nakamura T, Williams-Simons L, Westphal H. A human papillomavirus type 18 E6/E7 transgene sensitizes mouse lens cells to human wild-type P53-mediated apoptosis. Oncogene 1997;14:2991-8.

Pfeifer K, Leighton PA, Tilghman, SM. The structural H19 gene is required for transgene imprinting. Proc Natl Acad Sci USA 1996;93:13876-83.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. GDNF is required for kidney development and enteric innervation. Cold Spring Harbor Symp Quant Biol 1996;61:445-57.

Porter FD, Drago J, Xu Y, Huang SP, Lee EJ, Grinberg A, Cheema S, Massalas JS, Bodine D, Alt F, Westphal H. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. Development 1997;124:2935-44.

Sheng HZ, Bertuzzi S, Chiang C, Shawlot W, Taira M, Dawid I, Westphal H. Expression of murine Lhx5 suggests a role in specifying the forebrain. Dev Dyn 1997;208:266-77.

Sheng HZ, Moriyama K, Yamashita T, Li H, Potter SS, Mahon KA, Westphal H. Multistep control of pituitary organogenesis. Science 1997;278:1809-11.

Shores EW, Flamand V, Tran T, Grinberg A, Kinet J-P, Love PE. FcεRIγ can support T cell development and function in mice lacking endogenous TCR ζ chain. J Immunol 1997;159:222-30.

Shores EW, Love PE. TCR ζ-chain in T cell development and selection. Curr Opin Immunol 1997;9:380-89.

Shores EW, Tran T, Grinberg A, Sommers CL, Shen H, Love PE. Role of multiple TCR signaling motifs in selection of the T cell repertoire. J Exp Med 1997;185:893-900.

Suzuki H, Shinkai Y, Alt FW, Love PE, Singer, A. Commitment of immature $CD4^+CD8^+$ thymocytes to the CD4 lineage requires CD3 signaling but does not require expression of clonotypic TCR chains. J Exp Med 1997;186:17-24.

Tullio AN, Accili D, Ferrans VJ, Yu ZX, Takeda K, Grinberg A, Westphal, H, Preston YA, Adelstein RS. Nonmuscle myosin II-B is required for normal development of the mouse heart. Proc Natl Acad Sci USA 1997;94:12407-12.

Wadman IA, Osada H, Grütz GG, Agulnick AD, Westphal H, Forster A, Rabbitts TH. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the tal1, E47, GATA-1 and Ldb1/NLI proteins. EMBO J 1997;16:3145-57.

Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B, Olson L. Neurturin and GDNFR-b, novel proteins related to GDNF and GDNF-a with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J. Neurosci 1997;17:8506-19.

Yamashita T, Moriyama K, Sheng HZ, Westphal H. Lhx4, a LIM homeobox gene. Genomics 1997;44:144-6.

Zhang Z, Kundu GC, Yuan CJ, Ward JM, Lee EJ, DeMayo F, Westphal H, Mukherjee AB. Severe fibronectin-deposit renal glomerular disease in mice lacking uteroglobin. Science 1997;276:1408-12.

LABORATORY OF MOLECULAR EMBRYOLOGY

Alan P. Wolffe, Ph.D., Chief

The Laboratory has continued to make substantial progress in elucidating the molecular mechanisms responsible for the differentiation of germ cells in vertebrates, for establishing totipotency in the fertilized egg and for the subsequent differentiation of distinct cell lineages during early vertebrate development. The molecular basis of cellular determination during embryogenesis is a central problem of cell biology, and establishment and maintenance of stable differentiated states is essential to the development of an organism. When such controls break down, the cell may revert to a totipotent state, leading in turn to malignancy.

DEVELOPMENTAL REGULATION OF DIFFERENTIAL GENE EXPRESSION

The differentiated cell contains constellations of active and inactive genes. Research conducted by the Section of Molecular Biology, led by ***Alan Wolffe***, has contributed to an understanding of how both these states are established and how they coexist in the eukaryotic nucleus. During gametogenesis and embryogenesis, active chromatin can become repressed, and repressed chromatin activated, both reversibly. The reconstruction and experimental manipulation of these events demonstrate the interplay between chromosomal structure and specific transcription factors in controlling differential gene expression during vertebrate development.

Alan Wolffe

Histone Acetylation: Chromatin in Action. Chromatin is not a static entity in which DNA is packaged and forgotten by the molecular machines controlling transcription, replication, and repair. Instead, chromatin structure is dynamic, accommodating the need for DNA to partake in the various functions that require it as a template. Recent studies that showed some transcriptional coactivators to be histone acetyltransferases support an essential role for the post-translational modification of chromatin, particularly by targeted histone acetylation in transcriptional regulation. High levels of histone acetylation are correlated with gene activity and reduced levels with gene silencing. However, a causal relationship between histone acetylation and the relief of chromatin-mediated transcriptional repression had not been established prior to this work.

We have now shown in three distinct assays that histone acetylation directly promotes access of the transcriptional machinery to nucleosomal templates. In earlier work, we had demonstrated that acetylation of core histones in mononucleosomes containing a 5S rRNA gene would facilitate the access of TFIIIA to its recognition element within the gene. We have now extended this analysis using dinucleosomal templates competent for *in vitro* transcription. We find that acetylation of the core histones enhances both transcription initiation and elongation by RNA polymerase III. This effect is not dependent on an increased mobility of the core histone octamer with respect to DNA sequence. When linker histone is subsequently bound, we find both a reduction in nucleosome mobility and a repression of transcription. These effects of linker histone binding are independent of core histone acetylation, indicating that core histone acetylation does not prevent linker histone binding and the concomitant transcriptional repression. These studies are complemented by the use of a *Xenopus* egg extract competent both for chromatin assembly on replicating DNA and for RNA polymerase III transcription. Together, incorporation of acetylated histones and lack of linker histones facilitate transcription more than ten-fold in this system; however, they have little independent effect on transcription. Thus, core histone acetylation significantly facilitates transcription, but this effect is inhibited by the assembly of linker histones in chromatin, indicating that other, as yet unknown, mechanisms must facilitate the removal of histone H1 from chromatin if core histone acetylation is to facilitate transcription.

Sin Mutations of Histone H3 Influence Nucleosome Stability. In the yeast *Saccharomyces cerevisiae*, genetic evidence suggests that the activities of both histone acetyltransferases and molecular machines such as the SWI/SNF complex have roles in alleviating chromatin-mediated transcriptional repression. The connection between the SWI/SNF complex and chromatin lies in the genetic identification of suppressors of the requirement for the SWI/SNF complex for transcriptional activation in yeast. These Switch Independent (Sin) mutations are found in several structural components of chromatin.

We have investigated the structural and functional consequences for the nucleosome of Sin mutations in histone H3. We have directly tested the hypothesis that mutations in histone H3 leading to a SWI/SNF- independent (Sin) phenotype in yeast lead to nucleosomal destabilization. In certain instances, this is shown to be true; however, nucleosomal destabilization does not always occur. Topoisomerase-I-mediated relaxation of minichromosomes assembled with either mutant histone H3 or with wild-type H3, together with histones H2A, H2B, and H4, indicates that DNA is constrained into nucleosomal structures containing either mutant or wild-type proteins. However, nucleosomes containing particular mutant H3 molecules (R116-H, T118-I) are more accessible to digestion by micrococcal nuclease, and do not constrain DNA in a precise rotational position, as revealed by digestion with DNase I. This result establishes that Sin mutations in histone H3 located close to the dyad axis can destabilize histone-DNA contacts at the periphery of the nucleosome core. Other nucleosomes containing a distinct mutant H3 molecule (E105-K) associated with a Sin phenotype show very little change in nucleosome structure and stability compared with wild-type nucleosomes. Both mutant and wild-type nucleosomes continue to restrict the binding of either TBP/TFIIA or the RNA polymerase III transcription machinery. Thus, different Sin mutations in histone H3 alter the stability of histone-DNA interactions to varying extents in the nucleosome while maintaining the fundamental architecture of the nucleosome and contributing to a common Sin phenotype. Our results lend strong support to the concept that chromatin is a conformationally plastic and dynamic nucleoprotein complex. The SWI/SNF complex would be predicted to direct transitions in nucleosome stability similar to those seen in the Sin mutations in our analysis.

GENE REGULATION DURING EARLY EMBRYOGENESIS

Remodeling of Regulatory Nucleoprotein Complexes During Meiotic Maturation. Dramatic differences exist in the regulation of gene expression between oocytes and eggs. Within the oocyte, the maternal genome directs the complex differentiative process by which stores of macromolecules necessary for early development accumulate. Within an egg, these stores are utilized immediately following fertilization to initiate the assembly of the embryo. In striking contrast to the oocyte, the chromosomes of the vertebrate egg or embryo are usually transcriptionally silent at least until after the first cleavage division. The transition from an oocyte to an egg is a major developmental step, which is generally termed "meiotic maturation" in *Xenopus*.

In earlier work, we described the assembly of the *Xenopus* hsp70 promoter into a transcription complex that can be made dependent for activity on two *trans*-activators: heat shock transcription factor (HSF) and Gal4-VP16. We have now followed the transcriptional activity and nucleoprotein organization of the hsp70 promoter through meiotic maturation. Both transcriptional activity and the maintenance of the regulatory nucleoprotein complex on the hsp70 promoter during this developmental transition are found to be dependent on the abundance of transcriptional activators and on the efficiency with which the template is assembled into nucleosomes. The efficiency of chromatin assembly increases dramatically following meiotic maturation. Our results provide direct evidence for the regulation of gene expression through a mechanism in which the transcriptional machinery exists in a continual competition with chromatin proteins for association with regulatory DNA.

Regulated Transitions in Linker Histone Variants During Embryogenesis Control Cellular Competence. *In vitro* reconstitution of non-specific chromatin templates has led to the general conclusion that histone H1 can repress transcription. Experiments with natural chromosomal templates indicate, however, that the role of linker histones *in vivo* is much more selective. The first indication that a particular histone could contribute to the transcriptional control of a specific gene *in vivo* came from work in *Xenopus*. Histone H1 incorporation into the chromosomes of a somatic cell leads to the apparently specific activation of the oocyte-type 5S ribosomal RNA genes. Experiments, using a targeted ribozyme to specifically prevent the synthesis of histone H1 protein, demonstrated that H1 functions as a developmentally regulated gene-selective repressor during *Xenopus* embryogenesis. As embryogenesis proceeds, an oocyte-selective linker histone variant B4 is replaced by histone H1, and particular genes, including the oocyte-type 5S ribosomal RNA genes, are repressed. We have now

found that genes controlling muscle differentiation are also selectively repressed by histone H1. Interfering with histone H1 accumulation extends the period during embryogenesis in which cells of the mesoderm are competent to differentiate, which leads to a large increase in the amount of muscle tissue. Importantly, the vast majority of genes whose expression is essential to the continued viability of cells in the developing embryo are not activated by the inhibition of H1 protein accumulation. This result suggests that histone H1 is not a general repressor of transcription. Molecular analysis indicates that the extension of the competence of the cells of the mesoderm to respond to inductive signals correlates with the extension of expression of the master control regulatory protein MyoD. Our current studies examine the impact of histone H1 on the chromatin structure of the MyoD gene.

DNA Methylation as a Dominant Regulator of Gene Activity. The regulation of DNA methylation is required for differential expression of imprinted genes during vertebrate development. Earlier studies that monitored the activity of the Herpes simplex virus (HSV) thymidine kinase (tk) gene after injection into rodent cells have suggested that assembly of chromatin influences the methylation-dependent repression of gene activity. We have now examined the mechanism of methylation-dependent HSV tk gene regulation by direct determination of nucleoprotein organization during the establishment of a transcriptionally silenced state after microinjection of templates with defined methylation states into *Xenopus* oocyte nuclei.

The transcriptional silencing conferred by a methylated DNA segment is not immediate, as methylated templates are initially assembled into active transcription complexes. The eventual loss of DNase I hypersensitive sites and inhibition of transcription at the HSV tk promoter only occurs after several hours. Flanking methylated vector DNA silences the adjacent unmethylated HSV tk promoter, indicative of a dominant transmissible repression originating from a center of methylation. The resulting repressive nucleoprotein structure silences transcription in the presence of activators that are able to overcome repression of transcription by nucleosomes.

We conclude from these experiments that silencing of transcription by DNA methylation is achieved at the level of transcription initiation and involves the removal of transcriptional machinery from active templates. This transcriptional repression can occur by indirect mechanisms involving the time-dependent assembly of repressive nucleoprotein complexes, which are able to inhibit transcription more effectively than nucleosomes alone. We will make use of this system to explore the role of genome methylation in gene activity during early *Xenopus* development.

GENE REGULATION BY THYROID HORMONE

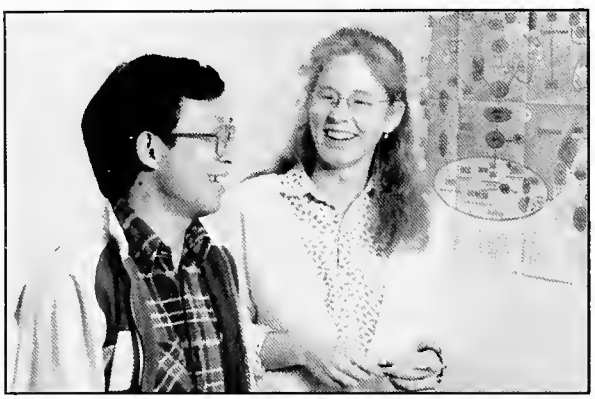
Transcriptional Regulation and Chromatin Disruption by Thyroid Hormone Receptors. ***Yun-Bo Shi***, who directs the Unit on Molecular Morphogenesis, and Alan Wolffe have collaborated on the molecular mechanisms by which the thyroid hormone receptor (TR) controls gene expression in chromatin. In earlier work, these investigators established that the TR makes very effective use of chromatin assembly to both activate and repress genes.

The superfamily of steroid and nuclear receptors are sequence-specific DNA binding proteins that regulate the transcription of genes containing their binding sites (TREs or thyroid hormone response elements for TRs) in a ligand-dependent manner. While TRs can bind to TREs as monomers and homodimers, they are most likely to function as heterodimers formed with RXRs (9-*cis* retinoic acid receptor). Earlier work has shown that TR/RXR heterodimers can bind to a TRE present in chromatin and regulate transcription in a T_3-dependent manner. We have now extended these studies by analyzing effects of placing different number of TREs at different locations in the T_3-responsive promoter. Our results have shown that TREs at a single location, independent of the exact number, cause similar extents of chromatin disruption, as measured by micrococcal nuclease digestion and supercoiling assays. On the other hand, multiple TREs at a single location have a greater effect on transcription, especially when located more than 500 bp away from the start site, than a single TRE at the same location. In addition, increasing the number of sites containing TREs, separated by more than 200 bp, increases chromatin disruption but not transcription. These results, taken together with our earlier studies, imply that chromatin disruption is necessary but not sufficient for T_3-dependent, TR/RXR-mediated transcriptional activation.

To understand the nature of TR/RXR interaction with the TRE in the TRβ promoter in the chromatin context, we have reconstituted *in vitro* the promoter region containing the TRE into a positioned nucleosome. We have determined the translational position of the histone octamer with respect to DNA sequence for a nucleosome containing the TRE and the rotational positioning of the double helix with respect to the histone surface. Histone

H1 incorporation into the nucleosome leads to an asymmetric protection against micrococcal nuclease cleavage of linker DNA relative to the nucleosome core, but has no effect on the rotational positioning of DNA, nor has it any significant consequence for the binding of TR/RXR heterodimer to nucleosomal DNA *in vitro*. Consistently, we have found that expression of histone H1 in oocytes has no significant effect on the regulation of TRβA gene transcription following microinjection into the oocyte nucleus. Finally, small alterations (3, 6 and 10 bp) in the translational positioning of the TRE in chromatin are without effect on the transcriptional activity of the TRβA gene, whereas a small change in the rotational position of the TRE (3 bp) relative to the histone surface reduces the binding of TR/RXR to the nucleosome and decreases transcriptional activation directed by TR/RXR. Our results indicate that the specific architecture of the nucleosome containing the TRE may have regulatory significance for expression of the TRβA gene.

Thyroid Hormone Receptor Function in Developing Embryos. Thyroid hormone is the causative agent of amphibian metamorphosis. To study the roles of TR and RXR during frog development, TRs and RXRs have been introduced either individually or together into developing *Xenopus* embryos. Phenotypic analysis has demonstrated that RXRs are critical for the developmental function of TRs. Precocious expression of TRs and RXRs together but not individually leads to drastic, distinct embryonic abnormalities, depending upon the presence or absence of thyroid hormone; and these developmental effects require the same receptor domains as those required for transcriptional regulation by TR/RXR heterodimers. More importantly, the overexpressed TR/RXR heterodimers faithfully regulate endogenous TH-response genes that are normally regulated by thyroid hormone only during metamorphosis. That is, they repress the genes in the absence of thyroid hormone and activate them in the presence of the hormone. On the other hand, the receptors have no effect on a retinoic acid (RA) response gene. Thus, retinoic acid and thyroid hormone-receptor-mediated teratogenic effects in *Xenopus* embryos occur through distinct molecular pathways, even though the resulting phenotypes share similarities.



Yun-Bo Shi and Mary Dasso

Cell-Cell and Cell-Extracellular Matrix Interactions During Thyroid Hormone Dependent Tissue Remodeling. Each metamorphosing organ consists of many different cell types. Some of them are in direct contact with each other. Others are separated by extracellular matrices (ECM). Proper interactions among these cells are likely to be important for tissue remodeling. Intestinal remodeling serves as an ideal system to study cell-cell and cell-ECM interactions. The larval intestine consists mostly of a single layer of epithelial cell organized in a tubular structure with little connective tissue or muscles. As the endogenous thyroid hormone concentration rises, the connective tissue starts to increase in thickness, and larval epithelial cells undergo apoptosis (programmed cell death). Concurrently, adult epithelial cells, whose origin is yet unknown, proliferate as cell islets. Around the time when cell death is mostly complete, adult epithelial cell differentiation is initiated. Within a few days, a multiple folded adult epithelium is developed, which is surrounded by elaborate connective tissue and muscle.

Among the thyroid hormone response genes that we have isolated during *Xenopus* intestinal remodeling are those encoding matrix metalloproteinases. Our earlier studies suggested that these genes are involved in regulation of cell-cell and cell-ECM interactions during metamorphosis. In particular, ECM remodeling, likely to involve these metalloproteinases, is associated with intestinal epithelial cell death and/or the proliferation and differentiation of adult cells, including cells of the connective tissue, muscle, and adult epithelium.

To investigate how thyroid hormone induces some cells to die while causing others to proliferate and differentiate during this process, an *in vitro* culture of intestinal epithelial cells and fibroblasts was established. With this system, we have shown that thyroid hormone can enhance the proliferation of both cell types. However, thyroid hormone also concurrently induces larval epithelial apoptosis. Studies with known inhibitors of mammalian cell death reveal both similarities and differences between amphibian and mammalian cell death. These investigations have implicated nucleases and ICE-like proteases (caspases) in TH-induced intestinal cell death, just as in mammalian apoptosis. Surprisingly, this TH-induced apoptosis is inhibited only by CsA but not by FK506, while both immunosuppressants block activation-induced apoptosis in T cells. Since TH exerts its effect primarily by regulating gene transcription through direct binding to nuclear thyroid hormone receptors,

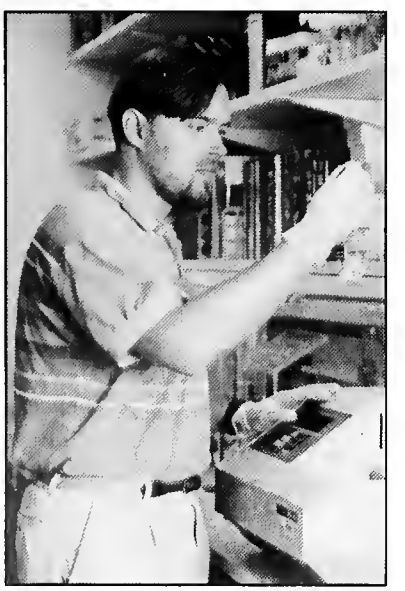
our results strongly suggest that, although they have similar functions in T cell receptor-mediated signal transduction process, only CsA, but not FK506, blocks another yet-unidentified step during the induction of apoptosis.

To investigate the effect of ECM, epithelial cells were cultured on plastic dishes coated with various ECM components, such as fibronectin and laminin. All ECM coatings enhance the survival of the cells in the absence of thyroid hormone and confer resistance to TH-induced apoptosis. Interestingly, they have little effect on cell proliferation. These results, together with gene expression analysis, reveal that T_3 appears to simultaneously induce different pathways that lead to specific gene regulation, proliferation, and apoptotic degeneration of the epithelial cells. Thus, our data provide an important molecular and cellular basis for the differential responses of different cell types to the endogenous T_3 during metamorphosis and support a role of extracellular matrix during frog metamorphosis.

THE ROLE OF SMC FAMILY PROTEINS AND ASSOCIATED FACTORS IN MITOTIC CHROMOSOME SEGREGATION

The Unit of Chromosome Structure and Function, directed by ***Alexander Strunnikov***, seeks to elucidate the molecular mechanics underlying the activity of SMC family of proteins in mitosis. This work follows four major directions: structure-function analysis of the SMC molecules using primarily mutational analysis of four SMC genes in bakers' yeast (*SMC1*, *SMC2*, *SMC3*, *SMC4*); analysis of cell-cycle specific distribution and regulation of individual SMC-proteins as the means to understand their specialization in eukaryotic cells; systematic search for the protein factors interacting with the SMC proteins genetically and biochemically in order to obtain information about composition of the SMC complex and biochemical activities associated with it; development of an *in vitro* assay for chromosome condensation employing chromatin as a substrate, to reveal fine mechanisms of SMC activity *in vivo*.

Relationship between Structural Integrity of the SMC Molecules and their Function. Mutational analysis of four SMC genes in bakers' yeast (*SMC1*, *SMC2*, *SMC3*, *SMC4*) reveals that both *SMC3* and *SMC4* genes are essential for viability, similar to the previously characterized *SMC1* and *SMC2* genes. Since the full genome of budding yeast has been sequenced, we know that there are only four SMC genes in this organism. That allowed us to launch a comparative study of all four SMC genes in the same organism. As all four genes have been shown to be essential for cell viability, it was possible to generate temperature-sensitive conditional-lethal alleles of all of them. Three new alleles, *smc2-8*, *smc3-1*, and *smc4-1*, have been compared with the previously published *smc1-2* allele with respect to their terminal morphologies, their chromosome loss and loss of viability, and their ability to be suppressed by dosage suppressors. Failure to segregate chromosomes in mitotic cell division under the restrictive temperature conditions has been detected in all alleles. All new mutant alleles are currently being sequenced to determine their position relative to the known putative structural elements of the SMC proteins: NTP-binding, coiled-coil, and DA-box regions.


Alexander Strunnikov

In mammalian organisms subjected to extensive genome sequencing efforts (man, mouse), four types of SMC genes can be found (*SmcA*, *SmcB*, *SCII*, and *SmcD*). This parallelism with the lower eukaryote *S. cerevisiae* suggests that the specialization of different SMC gene functions found in yeast may also exist in mammalian cells. The studies of SMC proteins in bigger cells have the clear advantage of higher resolution cytology in respect to chromosomes. To benefit from the detailed morphological studies feasible in higher eukaryotes, we initiated a project aimed at the genetic study of SMC genes in mouse cells (ES cells or primary fibroblasts). Recent improvements in gene targeting techniques and recycling of the selectable markers may allow us to assess the function of mouse SMC genes *in vivo*. The constructs for targeted knock-outs of three of mouse SMC genes, *SmcA*, *SmcB* and *SmcD* are being developed. The corresponding genomic clones of the 129/SvJ strain have been isolated as bacterial artificial chromosomes (BACs). Since we suspect that the disruption of these genes may be a lethal event in the absence of the ectopic expression of corresponding cDNA, the full-length

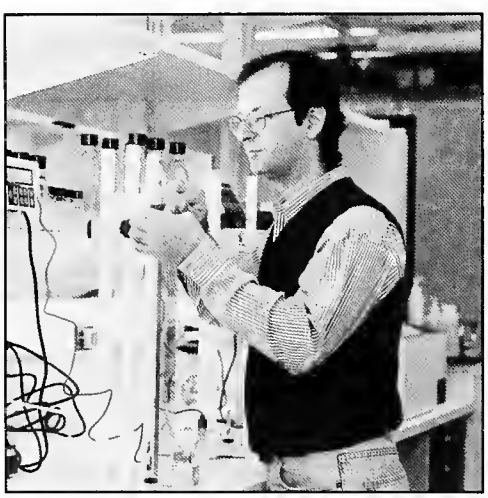
cDNAs for these genes are being assembled from the partial clones. We have a particular interest in the *SmcB* gene, which is located on the X chromosome, thus allowing a one-step knockout in diploid male cells. The main primary goal of these studies is to establish whether mouse SMC genes are essential for cell viability.

Search for the Protein Factors Interacting with the SMC Proteins. We have identified Mitotic Chromosome Determinant 1 (*MCD1*), as an evolutionary conserved yeast gene that is able to suppress the temperature-sensitive allele *smc1*-1 in a dosage-dependent manner. The Mcd1 protein is a subunit of the SMC complex in budding yeast, and we have established that the *MCD1* gene and its encoded protein are under tight cell-cycle regulation involving transcriptional induction in early S-phase, decrease in protein content in GO-M, and complete proteolysis after the completion of mitosis. This work demonstrates that Mcd1 is the first known factor to link chromosome condensation to sister chromatid cohesion in mitosis.

A GENETIC AND BIOCHEMICAL STUDY OF SILENCING

The Unit of Chromatin and Transcription, directed by ***Rohinton Kamakaka***, seeks to determine how distinct domains of gene expression are established and propagated in eukaryotes. Data from several laboratories have identified some of the proteins that are required for silencing of gene expression in *S. cerevisiae*, such as Orc, Rap1 and the Sir proteins. These studies have also revealed an intimate association between components of chromatin and silencing, thus setting the stage for a detailed investigation of the mechanism of silencing by the Sir proteins in the context of chromatin.

Identification of Novel Proteins Involved in Silencing. Macromolecular assemblies in biology are often sensitive to the relative dosage of different proteins components. Notable examples of this principle include the balance of histone protein levels that is critical for the proper segregation of chromosomes. To further our current understanding of the mechanism by which silenced domains are formed, we screened for gene products whose proper dosage is essential for silencing. A promoter fusion library over-expressing *S. cerevisiae* genes was used to screen for phenotypes associated with altered protein dosage in silent complexes. We identified a novel gene, *SAS10*, by the ability of its gene product Sas10p, to disrupt silencing when overproduced. The predicted Sas10p is strikingly rich in charged amino acids and is exclusively nuclear at all stages of the cell cycle. Overproduction of Sas10p causes derepression of mating type genes at both *HML* and *HMR*, as well as of the *TRP1* gene located near a telomere. Repressed genes not associated with silenced chromatin remain unaffected by overproduced Sas10p. The *SAS10* gene is essential for viability, and the termination point following Sas10p depletion is in the late S or Go/M phase of the cell cycle. Remarkably, Sas10p overproduction disrupts silencing even under conditions that bypass the requirement for Sir proteins, Orc, and Rap1p in silencing. Therefore, based on these observations, we favor the model in which Sas10p overexpression affects the establishment and inheritance of the silent state. Further experiments are in progress to isolate temperature-sensitive alleles of *SAS10* to determine precisely its role in silencing.


Rohinton Kamakaka

Suppressors of a Defective Silencer. Several lines of evidence suggest that DNA replication and the cell cycle play a role in the transcriptional silencing of *HMR* and *HML*. While the S-phase event that is required for establishment of the silent state remains unknown, efficient establishment and inheritance do require the silencer elements and Sir1p, and may be a consequence of replication-associated chromatin assembly. To learn more about the mechanism of establishment of the repressed state, we identified mutants that could restore silencing in strains containing a defective silencer. A large number of suppressors were identified (*CDC7, POL30, CDC44, Pol2, CLN3, CLB5, CDC45, MEC2*), and all these mutants could be classified as those that are required either for progression through the cell cycle or for post-replicative repair. In principle, these suppressor mutants could restore silencing by a number of different mechanisms: bypassing the need for a silencer; altering a process that functions through the silencer; or altering the proteins that mediate the effect of the silencer. We

are investigating the possibility that these mutants restore silencing by creating defects in the machinery that normally monitors replicated DNA prior to mitosis.

ANALYSIS OF THE S-PHASE CHECKPOINT IN HIGHER EUKARYOTES

The Unit on Cell Cycle Regulation, directed by ***Mary Dasso*** seeks to discover the mechanism by which cells transmit and interpret signals that coordinate nuclear activities with each other and with the cell cycle. In particular, we have focused on understanding the control of cell division with respect to the completion of DNA replication, since mitosis is inhibited by signals from the nucleus until DNA synthesis is complete. We are pursuing two molecular approaches to this problem. First, we are investigating the Ran GTPase pathway, which is required for nuclear transport and for coordination of nuclear activities to the cell cycle. Second, we are examining the role of *Rae1*, another gene product that is required for both nuclear transport and cell cycle control at mitosis.

Modification of RanGAP1 and RanBP2 by SUMO-1. Ran is a small GTPase of the Ras superfamily that is essential for nuclear transport, for mRNA processing, for maintenance of structural integrity of nuclei, and for cell cycle control. The best characterized role of Ran is in nuclear protein import, and multiple lines of evidence suggest that GTP hydrolysis by Ran is required to sustain both active protein import and export. Components of the Ran GTPase pathway are also required to regulate the entry into mitosis with respect to the completion of DNA replication. Experiments in the *Xenopus in vitro* system have suggested that Ran's role in regulating mitosis is distinct from its role in nuclear transport: *Xenopus* egg extracts mimic cell cycle transitions of the early embryo, allowing an examination of the effects of mutant Ran proteins on the regulation of mitosis. In cycling extracts, mutant Ran proteins block the activation of cyclin B/p34^{cdc2} as a mitotic kinase in the absence of nuclear DNA, indicating that Ran regulates mitosis in a manner that is independent of nuclear transport. Another protein that has been implicated in regulating the onset of mitosis is Ubc9p, a nuclear protein with homology to E2 ubiquitin-conjugating enzymes. In *S. cerevisiae*, repression of Ubc9p synthesis blocks cell cycle progression in late Go or early M phase, and results in the stabilization of B-type cyclins. However, Ubc9p is not able to ubiquinate mitotic cyclins *in vitro*, and it is unlikely to directly modify them for destruction during the cell cycle.

We have observed a complex of three Ran-interacting proteins in *Xenopus* extracts that is independent of the nucleotide binding state of Ran. This complex includes p340^{RanBP2}, the *Xenopus* homolog of RanBP2/Nup358, p88^{RanGAP1}, a modified form of RanGAP1/Fug1, and p18^{Ubc9} , the *Xenopus* homolog of Ubc9p. Mammalian RanBP2/Nup358 is a large nucleoporin with a leucine-rich domain, four RanBP1-related domains, a region of cyclophilin homology, and eight zinc fingers. RanBP2/Nup358 is thought to be the site of protein import substrate docking at the nuclear pore prior to translocation. RanGAP1/Fug1 is a GTPase activating protein (GAP) for Ran. p88^{RanGAP1} arises when unmodified RanGAP1, also known as p65^{RanGAP1} , is conjugated with a small ubiquitin-like protein, SUMO-1. We have observed that p88^{RanGAP1} is active as a GAP for Ran, and modification of RanGAP1 appears to be coupled to its association with RanBP2, since we do not observe unmodified RanGAP1 in anti-p340^{RanBP2} immunoprecipitates.

We further characterized the conjugation of SUMO-1 to RanGAP1 and other substrates *in vitro*. We found that SUMO-1 undergoes proteolytic processing prior to conjugation. Reticulocyte lysates lack the ability required to accomplish this proteolytic processing, but this activity is abundant in *Xenopus* egg extracts. Glutathione S transferase-SUMO-1 (GST-SUMO-1) fusion proteins associate tightly with p18^{Ubc9} in *Xenopus* egg extracts. Furthermore, p18^{Ubc9} forms a thioester linkage with processed GST-SUMO-1, consistent with the idea that p18^{Ubc9} acts as a conjugating enzyme for SUMO-1. Under the same conditions, we do not see association between GST-ubiquitin and p18^{Ubc9}, nor have we been able to demonstrate thioester formation between ubiquitin and p18^{Ubc9}. These results provide strong evidence that p18^{Ubc9} acts as an E2-like enzyme for SUMO-1 conjugation. We have mapped the domain of RanBP2 that associates with p18^{Ubc9} by expressing fragments of the mammalian RanBP2 in reticulocyte lysates and examining their capacity to associate with a p18^{Ubc9}. We find that p18^{Ubc9} associates very specifically with the internal repeat domain of RanBP2. Interestingly, we also find that this domain is a substrate for GST-SUMO-1 conjugation in egg extracts. Our results provide direct evidence that the pathway for SUMO-1 conjugation may be mechanistically similar to the ubiquitin conjugation pathway, but that it uses a distinct set of enzymes and regulatory mechanisms. Our results also provide the second known substrate for SUMO-1 conjugation, and strongly suggest that SUMO-1 modification is involved in regulating

multiple components of the nuclear transport machinery. These results may suggest a link between nuclear transport and the degradation of B-type cyclins.

Balance of RanBP1 and RCC1 is Critical for Nuclear Assembly and Nuclear Transport. RanBP1 is a guanine nucleotide dissociation inhibitor (GDI) for GTP-Ran. Like Ran, RanBP1 is ubiquitously expressed and highly conserved across species. RanBP1 acts a cofactor for RanGAP1, increasing Ran's *in vitro* rate of GAP-mediated hydrolysis by an order of magnitude. RanBP1 has a high affinity for GTP-bound Ran and a low affinity for GDP-bound Ran. RanBP1 does not interact strongly with RCC1, Ran's guanine nucleotide exchange factor, in the absence of Ran. However, when Ran is in a nucleotide-free state, RanBP1 forms a stable heterotrimeric complex with RCC1 and Ran. This complex rapidly dissociates with the addition of magnesium and GTP, but not of GDP. The association between GTP-Ran and RanBP1 stabilizes the bound nucleotide and inhibits further RCC1-induced exchange.

In order to examine RanBP1's role in nuclear assembly *in vitro*, we cloned a *Xenopus* RanBP1 homolog and used it to generate recombinant RanBP1 protein and anti-RanBP1 antibodies. We removed RanBP1 from *Xenopus* egg extracts by serial depletion with affinity purified anti-RanBP1 antibodies. Surprisingly, immunodepletion of RanBP1 resulted in co-depletion of RCC1, suggesting that RanBP1 and RCC1 can form a stable complex in extracts. Nuclei formed in extracts lacking both proteins (co-depleted extracts) do not exhibit defects in assays of assembly, DNA replication, or nuclear transport. Nuclei from co-depleted extracts also enter mitosis normally in response to the addition of recombinant cyclin B protein. Addition of either recombinant RanBP1 or RCC1 to co-depleted interphase extracts blocks nuclear assembly, nuclear transport, and DNA replication, a block that can be reversed further addition of RCC1 or RanBP1, respectively. Although the abnormal nuclei formed in extracts lacking either RanBP1 or RCC1 appear to be morphologically similar, their defects can be distinguished by their response to exogenous mutant Ran proteins. Our results demonstrate that little, if any, RanBP1 or RCC1 are required for interphase nuclear functions in the absence of the other protein. However, the results also suggest that the balance of RCC1 and RanBP1 is normally critical for proper nuclear assembly and function.

***Rae1* in *Xenopus* Egg Extracts.** The *rae1*-1 mutation in *S. pombe* causes a rapid inhibition of mRNA export from the nucleus, as well as a G_2/M phase arrest and a loss of cytoskeletal integrity. The cell cycle arrest in *Rae1*-1 mutants is independent of inhibitory tyrosine phosphorylation on $p34^{cdc2}$. *Rae1*-1 mutants also show defects in their response to ultraviolet irradiation, entering mitosis prematurely after UV treatment at the permissive temperature. The cell cycle execution point for *Rae1* occurs late in the G_2/M transition, after the translational execution point. This late execution point may indicate that the *Rae1* requirement at the G_2/M transition is independent of *Rae1*'s role in export of mRNAs encoding cell cycle regulators. Instead, it appears more likely that *Rae1*p is either required for the nuclear transport of another cell cycle regulator or regulators, or that it has a role in the induction of mitosis that is independent of its role in nuclear transport.

We investigated the behavior of a *Xenopus* Rae1 protein, a protein that is likely to be a functional homolog of yeast Rae1, since it is able to complement *Rae1*-1 mutants. We have found that *Xenopus* Rae1 is post-translationally modified. Gel filtration analysis indicates that this modification regulates the association of Rae1 with a high molecular weight complex in egg extracts. We find that three proteins are specifically associated with GST-Rae1, but fail to associate with a biologically inactive GST-Rae1 mutant that contains an eight-amino-acid deletion. Future studies will explore the identity and function of these proteins.

LABORATORY OF MOLECULAR EMBRYOLOGY

Chief
Alan P. Wolffe, Ph.D.

Tenure-Track Investigators
Mary Dasso, Ph.D.
Rohinton Kamakaka, Ph.D.
Yun-Bo Shi, Ph.D.
Alexander Strunnikov, Ph.D.

Senior Fellows
Hui Ge, Ph.D.
Dmitry Guschin, Ph.D.
Robert Kimmel, M.D.
Hisato Saitoh, Ph.D.

Pre-Doctoral Fellows
Christopher Adams
Vivia Liang
Maria Patente

Post-Doctoral Fellows
Juana Barcelo, Ph.D.
Sashko Damjanovski, Ph.D.

Nadine Darwiche, Ph.D.
Namrita Dhillon, Ph.D.
David Donze, Ph.D.
Lita Freeman, Ph.D.
Michael Herrler, Ph.D.
Peter Jones, Ph.D.
Petr Kalab, Ph.D.
Nikola Kaludov, Ph.D.
Jyoti Khadake, Ph.D.
Nobuaki Kikyo, Ph.D.
Nicoletta Landsberger, Ph.D.
Qiao Li, M.D., Ph.D.
Robert Pu, Ph.D.
Laurent Sachs, Ph.D.
Takashi Sera, Ph.D.
John Strouboulis, Ph.D.
Yuan Su, M.D.
Gert Veenstra, Ph.D.
Paul Wade, Ph.D.
Karen Wasserman, Ph.D.
Jiemin Wong, Ph.D.
Janet Yee, Ph.D.

Guest Researchers
Yoshiaki Azuma, Ph.D.
Esteban Ballastar, Ph.D.
Simon Chandler, Ph.D.
Trevor Collingwood, Ph.D.
Anne Gegonne, Ph.D.
Mark Hirst, Ph.D.
Axel Imhof, Ph.D.
Qing Li, M.D.
Yuanzheng Si, M.D.
Thomas Spencer, Ph.D.
Jamshed Tata
Mark Thomas, Ph.D.
Guo-Liang Yu, Ph.D.

ANNUAL BIBLIOGRAPHY

Bharathi A, Ghosh A, Whalen WA, Yoon JH, Pu R, Dasso M, Dhar R. The human *RAE1* gene is a functional homologue of *Schizosaccharomyces pombe rae1* gene involved in nuclear export of Poly(A)$^+$ RNA. Gene 1997;198:251-8.

Denver RJ, Pavgi S, Shi Y-B. Thyroid hormone-dependent gene expression program for *Xenopus* neural development. J Biol Chem 1997;272:8179-88.

Dimitrov SI, Wolffe AP. Fine resolution of histones by two dimensional polyacrylamide gel electrophoresis: developmental implications. Methods 1997;12:57-61.

Guacci V, Koshland D, Strunnikov AV. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of *MCD1* in *S. cerevisiae*. Cell 1997;91:47-57.

Imhof A, Yang XJ, Ogryzko VV, Nakatani Y, Wolffe AP, Ge H. Acetylation of general transcription factors by histone acetyltransferases: identification of a major site of acetylation in TFIIEβ. Curr Biol 1997;7:689-92.

Ishizuya-Oka A, Stolow MA, Ueda S, Shi Y-B. Temporal and spatial expression of an intestinal Na^+/PO_4^{3-} cotransporter correlates with epithelial transformation during thyroid hormone-dependent frog metamorphosis. Dev Genet 1997;20:53-66.

Ishizuya-Oka A, Ueda S, Shi Y-B. Temporal and spatial regulation of a putative transcriptional repressor implicates a role during thyroid hormone-dependent organ transformation. Dev Genet 1997;20:329-37.

Kamakaka RT. Silencers and locus control regions: opposite sides of the same coin. Trends Biochem Sci 1997;22:124-8.

Kamakaka RT, Bulger M, Kaufman PD, Stillman B, Kadonaga JT. Postreplicative chromatin assembly by Drosophila and human chromatin assembly factor 1. Mol Cell Biol 1996;16:810-7.

Kass SU, Landsberger N, Wolffe AP. DNA methylation directs a time-dependent repression of transcription initiation. Curr Biol 1997;7:157-165.

Kass SU, Pruss D, Wolffe AP. How does DNA methylation repress transcription? Trends Genetics 1997;13:444-9.

Kass SU, Wolffe AP. DNA methylation, the nucleosome and the inheritance of chromatin structure and function. In: Cardew G, ed. Novartis Foundation Symposium No 214, Epigenetics. London: John Wiley, 1997;22-45.

Koshland D, Strunnikov A. Mitotic chromosome condensation. Annu Rev Cell Dev Biol 1996;12:305-33.

Kurumizaka H, Wolffe AP. Sin mutations of histone H3: influence on nucleosome core structure and function. Mol Cell Biol 1997;17:6953-69.

Landsberger N, Wolffe AP. Remodeling of regulatory nucleoprotein complexes on the *Xenopus* hsp70 promoter during meiotic maturation of the *Xenopus* oocyte. EMBO J 1997;16:4361-73.

Meric F, Matsumoto K, Wolffe AP. Regulated unmasking of *in vivo* synthesized maternal mRNA at oocyte maturation: a role for the chaperone nucleoplasmin. J Biol Chem 1997;272:12840-6.

Pruss D, Bavykin SG. Chromatin studies by DNA-protein crosslinking. Methods 1997;12:36-47.

Pu RT, Dasso M. The balance of RanBP1 and RCC1 is critical for nuclear assembly and nuclear transport. Mol Biol Cell 1997;10:1955-70.

Puzianowska-Kuznicka M, Wong J, Kanamori A, Shi Y-B. Functional characterization of a mutant thyroid hormone receptor in Xenopus laevis. J Biol Chem 1996;271:33394-403.

Puzianowska-Kuznicka M, Damjanovski S, Shi Y-B. Both TR and RXR are required to efficiently mediate the effects of thyroid hormone on embryonic development and specific gene regulation in Xenopus laevis. Mol Cell Biol 1997;17:4738-49.

Saitoh H, Pu RT, Cavenagh M, Dasso M. RanBP2 associates with Ubc9p and a modified form of RanGAP1. Proc Natl Acad Sci USA 1997;94:3736-41.

Saitoh H, Pu R, Dasso M. SUMO-1: wrestling with a new ubiquitin-related modifier. Trends Biochem Sci 1997;262:374-6.

Shi Y-B. Cell-cell and cell-ECM interactions in epithelial apoptosis and cell renewal during frog intestinal development. Cell Biochem Biophys 1997;27:179-202.

Shi Y-B, Ishizuya-Oka A. Auto-activation of Xenopus TRβ genes correlates with larval epithelial apoptosis and adult cell proliferation. J Biomed Sci 1997;4:9-18.

Shi Y-B, Shi Y, Scott D, Xu Y, eds. Programmed cell death, New York: Plenum Publishing Corp, 1997;171.

Shi Y-B, Ishizuya-Oka A. Activation of matrix metalloproteinase genes during thyroid hormone-induced apoptotic tissue remodeling. In: Shi Y-B, Shi Y, Scott D, Xu Y, eds. Programmed cell death. New York: Plenum Publishing Corp, 1997;13-26.

Shi Y-B, Wang R-X, Shi Y-F. Apoptosis: the molecular mechanism. Life Sci 1996;8:8-12.

Steinbach O, Wolffe AP, Rupp R. A transition from maternal to somatic linker histones causes loss of mesodermal competence in *Xenopus laevis*. Nature 1997;389:406-12.

Stolow MA, Bauzon DD, Li J, Sedgwick T, Liang VC-T, Sang QA, Shi Y-B. Identification and characterization of a novel collagenase in Xenopus laevis: Possible roles during frog development. Mol Biol Cell 1996;7:1471-83.

Stolow MA, Ishizuya-Oka A, Su Y, Shi Y-B. Gene regulation by thyroid hormone during amphibian metamorphosis: implications on the role of cell-cell and cell-extracellular matrix interactions. Am Zool 1997;37:195-207.

Su Y, Shi Y, Shi Y-B. Cyclosporin A but not FK506 inhibits thyroid hormone-induced apoptosis in *Xenopus* tadpole intestinal epithelium. FASEB J 1997;11:559-65.

Tyler JK, Bulger M, Kamakaka RT, Kobayashi R, Kadonaga JT. The p55 subunit of Drosophila chromatin assembly factor 1 is homologous to a histone deacetylase-associated protein. Mol Cell Biol 1996;16:6149-59.

Ura K, Kurumizaka H. Transcriptional regulation in chromatin. Exp Med 1997;14:78-85.

Ura K, Kurumizaka H, Dimitrov S, Almouzni G, Wolffe AP. Histone acetylation: influence on transcription by RNA polymerase, nucleosome mobility and positioning, and linker histone dependent transcriptional repression. EMBO J 1997;16:2096-107.

Wade PA, Wolffe AP. Histone acetyltransferases in control. Curr Biol 1997;7:R82-R84.

Wade PA, Pruss D, Wolffe AP. Histone acetylation: chromatin in action. Trends Biochem Sci 1997;22:128-32.

Wolffe AP. Chromatin and gene regulation at the onset of embryonic development. Reprod Nutr Dev 1996;36:581-606.

Wolffe AP. Chromatin structure. Adv Mol Cell Biol 1997;24:363-414.

Wolffe AP. Histone modification and transcription competence. In: van Driel R, Otte A, eds. Nuclear organization, chromatin structure and gene expression. Oxford: Oxford University Press, 1997;40-57.

Wolffe AP. Histones, nucleosomes and the role of chromatin structure in transcription control. Biochem Soc Trans 1997;25:354-8.

Wolffe AP. Transcriptional control. Sinful repression. Nature 1997;387:16-7.

Wolffe AP. Transcriptional regulation in a chromatin environment. In: Nicolini C, ed. Genome structure and function. Dordrecht: Kluwer, 1997;83-110.

Wolffe AP, Khochbin S, Dimitrov S. What do linker histones do in chromatin? Bioessays 1997;19:249-55.

Wolffe AP, Nightingale KP, Ura K. Nucleosomes and transcription. Nucleic Acids Mol Biol 1997;11:275-90.

Wolffe AP, Ura K. Transcription of dinucleosomal templates. Methods 1997;12:10-19.

Wolffe AP, Wong J, Li Q, Levi B-Z, Shi Y-B. Three steps in the regulation of transcription by the thyroid hormone receptor: establishment of a repressive chromatin structure, disruption of chromatin and transcriptional activation. Biochem Soc Trans 1997;25:612-5.

Wolffe AP, Wong J, Pruss D. Activators and repressors: making use of chromatin to regulate transcription. Genes Cells 1997;2:291-302.

Wong J, Shi Y-B, Wolffe AP. Determinants of chromatin disruption and transcriptional regulation instigated by the thyroid hormone receptor: hormone regulated chromatin disruption is not sufficient for transcriptional activation. EMBO J 1997;16:3158-71.

LABORATORY OF MOLECULAR GENETICS

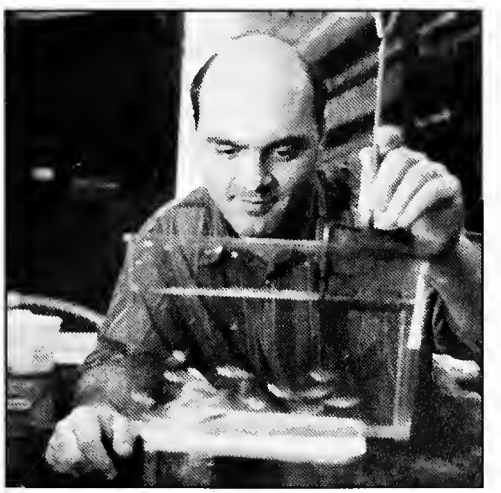
Igor B. Dawid, Ph. D., Chief

In the past year, the members of the Laboratory have extended their studies on mechanisms of gene regulation in different systems and in different developmental and physiological conditions. These problems were explored for bacterial and animal viruses, in the responses of bacterial cells to environmental conditions, and during development of multicellular organisms. A particular focus of the research in the Laboratory is the question of pattern formation and its genetic control in different animals. In the recent past, substantial progress has been made in this area of research, allowing the study of classical questions of embryology at the cellular and molecular level. Work in this Laboratory contributes to this progress in the areas of axis establishment, cell-cell signaling in mesoderm and neural induction, and the elaboration of regulatory circuits during embryogenesis.

This Laboratory has led the way within the NIH Intramural Research Program in initiating research on zebrafish, with Igor Dawid's group working in this area for the past five years. This system has risen rapidly in importance during the past several years, and especially since the publication of an entire issue of the journal Development in December 1996 devoted to the description of several hundred new developmental mutations. This set of mutants, together with growing molecular and embryological information and a budding genome project, make the zebrafish a major system for the mechanistic study of vertebrate development. Thus, the most significant event in the life of the Laboratory of Molecular Genetics (LMG) during the past year was the recruitment of two investigators, whose programs use the zebrafish model system to study vertebrate development, which will greatly expand the scope of zebrafish studies within the Laboratory and the Institute. Correspondingly, a substantial investment into renovations and construction of animal facilities is under way, which will eventually provide the three groups in the Laboratory with up to 5,000 fish tanks, allowing the execution of genetic experiments on an effective scale.

ORGANOGENESIS IN THE VERTEBRATE EMBRYO

Brant Weinstein is one of two investigators newly appointed in the Laboratory. The goal of this group, the Unit on Vertebrate Organogenesis, is to understand how specification and patterning of internal organs is accomplished during vertebrate embryogenesis, with a particular focus on the origins of the vascular and hematopoietic components of the circulatory system. The zebrafish is ideally suited for studying the developing circulatory system because of the physical accessibility and remarkable optical clarity of zebrafish embryos. Together with former colleagues, we obtained and characterized a number of zebrafish mutants that cause specific defects in the early development of either blood vessels or blood cells. We also developed new experimental techniques for studying circulatory development in zebrafish embryos, including embryonic blood cell transfusion and 3-D confocal microangiography. Using these and other genetic and experimental tools, we are attempting: (1) to elucidate the embryonic origins of vascular endothelial cells and of hematopoietic stem cells and determine how these two lineages are related; and (2) to understand how the patterning of the developing vasculature is directed.



Brant Weinstein

Vascular Patterning Genes Uncovered by Specific Zebrafish Mutations. Previous work had shown that single gene defects, such as the *gridlock* mutation, can cause defined, localized defects in the patterning of blood vessels. We are now attempting to further define the nature and consequences of *gridlock* and other potential vascular patterning mutations using a variety of genetic, experimental embryologic, molecular, and molecular genetic methods.

Trunk Axial Vessel Specification and Patterning. In addition, the dorsoventral specification and patterning of the major axial trunk vessels (axial artery or aorta and axial vein) will be studied. We have obtained evidence supporting a "two-signal model" for axial vessel formation, reminiscent of that invoked to explain the dorsoventral patterning of the neural tube, and we are investigating the nature and activities of these signals.

Hematopoietic Progenitor Cells. We will also study hematopoiesis-specific mutations, with emphasis on several early-acting mutations that may cause defects in the formation of hematopoietic progenitor cells.

PATTERNING NEUROGENESIS

The second newly appointed investigator, ***Ajay Chitnis***, joined this Laboratory in October to head the Unit on Vertebrate Neural Development. Chitnis is interested in understanding cell-cell interactions that determine the spatial distribution of early neurons in the spinal cord. In *Xenopus* and zebrafish embryos, the distribution of these neurons is relatively simple; at the early neural plate stage, they are found in a salt-and-pepper pattern in three longitudinal "proneuronal domains" on either side of the midline. Previously, Chitnis showed that lateral inhibition mediated by *Xenopus* homologs of *Notch* and *Delta* helps to select cells that become neurons within these longitudinal domains in the neural plate. Chitnis is continuing to explore the role of neurogenic genes in determining the pattern of neurons by analyzing a zebrafish mutant, *mindbomb,* which is characterized by an overproduction of neurons within proneuronal domains. Furthermore, to identify genes that determine how proneuronal domains are established, he has undertaken a genetic screen to identify mutants with an altered pattern of early neurons. His laboratory is now using a combination of cellular, molecular and genetic approaches to characterize and analyze mutants identified in this screen.

Ajay Chitnis

EMBRYONIC INDUCTION AND AXIS SPECIFICATION

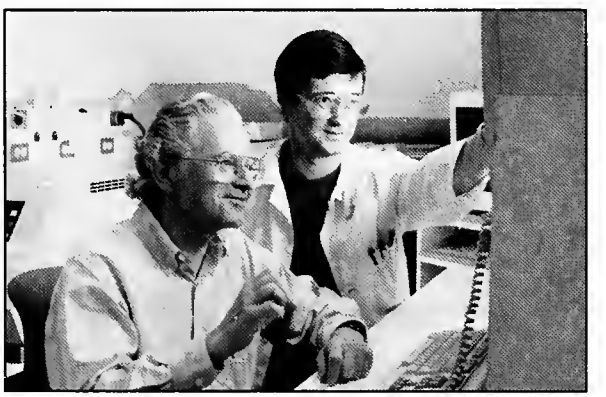
Zebrafish Studies. The Section on Developmental Biology, headed by ***Igor Dawid***, has conducted studies on molecular mechanisms controlling early vertebrate development for some time. The laboratory has used *Xenopus* as an experimental system for an extended period, and for the past five years has added the zebrafish as a model to take advantage of the new opportunities offered by this system. Recent work with zebrafish dealt with several questions, but one of primary current interest concerns nodal-related (Ndr) factors. These factors constitute a subgroup of the TGF-β family, which includes numerous growth and differentiation factors that regulate many aspects of development in all animals. The founding member of the group, nodal, is known to be expressed in the node (organizer equivalent) of the mouse and is required for mesoderm formation during gastrulation. While only one mouse nodal has been found, we isolated three *ndr* genes from zebrafish, two of which were studied in detail. Both *ndr1* and *ndr2* are expressed in the early embryo in the shield (organizer equivalent in fish), and both have a dorsalizing effect in embryogenesis. However, injection experiments in *Xenopus* animal explants indicate that Ndr1 behaves as a dorsal mesoderm inducer, similar to activin, whereas Ndr2 does not induce mesoderm but acts as a dorsalizing and neuralizing factor during gastrulation. This shows an interesting distinction in the function of two closely related factors. In later development, Ndr2 is expressed in anterior axial mesoderm; the possibility that *ndr2* corresponds to one of the mutations affecting midline structures that have been described in zebrafish is currently being tested. In addition, *ndr2* is transiently expressed in an asymmetric fashion on the left side of the lateral plate and in a region of the forebrain. The involvement of nodal-related factors in left-right asymmetry has been proposed in other animals, but asymmetry in the forebrain has not previously been reported for any gene.

***Xenopus* Studies.** In *Xenopus*, mutation genetics is not an effective tool, but reverse genetics is. Microinjected synthetic RNA directs the efficient expression of proteins in the embryo, allowing an assessment of the protein's

function *in vivo*. For several years, we have studied the role of the LIM homeodomain protein Xlim-1 in organizer function; these studies, together with work by others in the mouse, indicated that Xlim-1 is important in head formation. A method to study this question in *Xenopus* emerged recently when Jim Smith and colleagues introduced a general method for creating dominant-negative forms of transcription factors. Starting from an activating factor, one replaces the activation domain with the repressor domain from the *Drosophila* protein Engrailed; the resulting fusion protein can inhibit the normal function of the parent protein. This approach was successfully applied to Xlim-1. When the Xlim-1/enR construct is expressed on the dorsal side of the embryo, it inhibits head formation, leading to headless or microcephalic embryos. While this result is expected, it is particularly useful in allowing one to carry out epistasis studies. By coinjection of the appropriate constructs, it could be shown that the homeodomain protein Siamois acts upstream of Xlim-1 in organizer formation, whereas the signaling factor Chordin acts downstream and in parallel with Xlim-1. Thus, this approach should help in working out the regulatory networks that control axis formation and the shaping of the body pattern during gastrulation.

REGULATION OF SPATIAL ORGANIZATION AND TERMINAL DIFFERENTIATION IN VERTEBRATE EMBRYOS

The Section on Vertebrate Development, headed by ***Tom Sargent***, is seeking answers to two questions in developmental biology: (1) how cells sense their location in vertebrate embryos, enabling them to correctly migrate and establish proper connections and tissue boundaries; and (2) what the molecular mechanisms are that control terminal differentiation of cells in specific tissues. These problems are approached at the molecular level by analyzing the function and biochemistry of Eph-class receptor tyrosine kinases (RTKs) in *Xenopus* embryos and Distal-less homeodomain genes in mouse epidermis, respectively.



Igor Dawid (left) and Tom Sargent

Eph-Class RTKs. This project focuses on EphA4 (previously called "Pagliaccio" or "SEK1"), which is expressed in migrating mesodermal and neural crest cells. We have established a link between activation of this receptor and down-regulation of cell-cell adhesion in early frog embryos. We find that this effect appears to be mediated by the intracellular domain of one or more cadherins, and may require the src-related kinase c-fyn as an intermediate. Current work focuses on identifying other components of this signal transduction pathway. We are also carrying out experiments in which EphA4 activity is manipulated in *Xenopus* embryos to test for the involvement of this receptor in guiding migrating mesoderm and neural crest cells. This work is providing an important insight into the molecular biology and signal transduction of Eph-class receptors, which are essential factors in the development of all vertebrate embryos.

Dlx3 in Epidermal Differentiation. The model system used to address the problem of terminal differentiation is the epidermis. Specifically, we have found that the homeobox gene *Distal-less*-3 (*Dlx*3) is an important player in regulating differentiation of epidermal cells in the mouse. This process goes on continually, and requires precise coordination of gene activation and inactivation, as the stem cell, located near the dermis, gradually evolves into the crosslinked, lipid-containing cornified cell at the skin surface. *Dlx*3 appears to positively regulate this process; when it is prematurely activated in transgenic mice, the final steps in the epidermal developmental program are initiated immediately, resulting in highly abnormal and dysfunctional skin. Together with collaborators, we are currently dissecting the biochemical properties of Dlx3, and have identified both activating and repressive domains in the protein. We are also searching for genes that are direct regulatory targets of *Dlx*3, using a retrovirally-transduced, conditionally activated version of this homeoprotein in murine keratinocytes. We have knocked out the *Dlx*3 gene in the mouse, and find that this results in a lethal phenotype about halfway through gestation (day 11). *Dlx*3 is expressed at high levels in the placenta, and this tissue is abnormal in embryos lacking the *Dlx*3 gene. This strongly implicates *Dlx*3 in placental differentiation. From these results, it is clear that Dlx3 is an important regulatory protein in development, in the epidermis as well

as in other tissues. It should continue to be an excellent model system for examining homeodomain function and the control of tissue differentiation.

MOLECULAR GENETICS OF RNA-PROTEIN INTERACTIONS IN *DROSOPHILA*

The regulation of gene expression is a complex process that can occur at multiple levels, both transcriptionally and post-transcriptionally. The Unit on Protein-Nucleic Acid Interaction, directed by ***Susan Haynes***, studies *Drosophila* RNA binding proteins to understand how they regulate gene expression, particularly with respect to developmental and cell-type-specific regulation. These proteins contain RNA recognition motif (RRM) domains, which have been identified in many proteins involved in post-transcriptional regulation, and have been shown to mediate sequence-specific binding to RNA targets.

Functions of hnRNP Proteins. Our recent work has led to the identification of new mutants in three genes (*Hrb87F*, *Hrb98DE* and caz) that encode homologs of vertebrate nuclear pre-mRNA binding proteins (hnRNP proteins). HnRNP proteins associate with newly synthesized mRNA and may affect its splicing and transport. These studies on the new *Hrb* gene mutants have confirmed and extended their previous findings that neither gene is essential, but that doubly-mutant flies die as embryos. This synthetic lethality is specific for the *Hrb* genes, and suggests that they are functionally redundant. Extensive genetic studies of combinations of mutations in other *Drosophila* hnRNP proteins show that flies are viable as long as they have at least one functional *Hrb* gene. In contrast to the *Hrb* mutations, mutations in the caz hnRNP gene are lethal. Although there is a single copy of the caz gene in flies, vertebrates have at least five related proteins, which have been implicated in a variety of cellular processes. The caz mutations will be useful in assessing the role of this protein in *Drosophila*.

Jim Kennison and Susan Haynes

Functions of the NTS RRM Protein. In collaboration with the Mlodzik group at the EMBL, we are studying mutations in the *Nts* gene, which cause abnormal eye development and sterility in both sexes. Multiple transcripts are expressed throughout development, and arise by a combination of alternative splicing and polyadenylation. However, all the transcripts apparently encode the same protein, since anti-NTS antibodies detect only a single band on Western blots. This protein is present in the cytoplasm, is detectable throughout development, and is particularly enriched in ovaries. One of the most interesting features of NTS is that genetic interaction studies implicate it in signal transduction during eye development. This suggests that transcriptional regulation is not the only pathway by which signal transduction mechanisms affect gene expression. Rather, there may be direct affects on mRNA stability or translatability via interaction with cytoplasmic RNA binding proteins.

REGULATION OF HOMEOTIC GENES IN *DROSOPHILA*

The study of *Drosophila* as a model system has been particularly effective in elucidating the genetic basis of pattern formation. The discovery of homeotic genes, which control pattern formation, and their molecular characterization has revolutionized developmental biology in the last two decades. Homeotic genes control pattern formation through their spatially and temporally regulated expression during development. This, in turn, leads to regulated expression of multiple target genes. Thus, the issue of how expression of the homeotic genes is regulated is a major question in developmental genetics. ***Jim Kennison***, who leads the Unit on Drosophila Gene Regulation, has studied this question for some time, using the sophisticated genetic approaches available for *Drosophila* to identify genes required for homeotic gene function. These approaches use sensitized genetic backgrounds in which the homeotic genes are either only partially functional, or are expressed in the wrong tissues. In either case, the flies have altered phenotypes that are used to screen for new mutations that either increase or decrease the levels of homeotic gene function. These new mutations identify genes that regulate

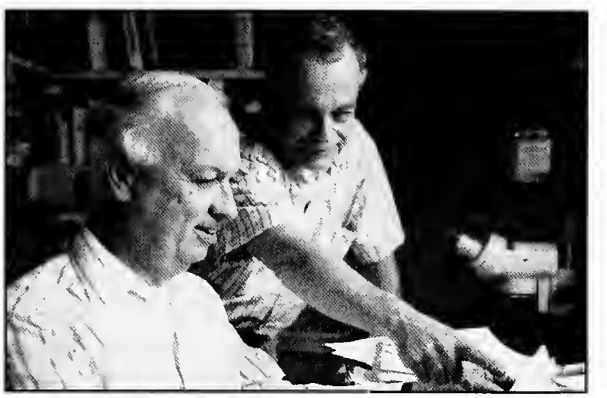
function of the homeotic genes at a variety of biochemical steps. Almost twenty new genes have been identified by this method.

Homeotic Gene Silencing. One of the most important aspects of the control of homeotic gene expression involves a silencing mechanism that maintains the homeotic genes in inactive states throughout development. Each homeotic gene is expressed only in a limited part of the fly, and expression in the wrong part of the fly causes the wrong structures to be formed, such as the formation of legs in place of antennae when silencing of the *Antennapedia* gene fails in the cells that form the head. Silencing is initiated by early acting repressors during embryogenesis and is maintained by a special group of repressor proteins called the Polycomb group. A new member of the Polycomb group that may encode a protein involved in the switch from the initiation to the maintenance phase of silencing has been identified in collaboration with J. Müller (Tübingen, FRG) and M. Bienz (MRC, Cambridge, UK). Studies on the structural elements required for silencing within the homeotic genes has suggested that silencing occurs by a chromosomal looping mechanism rather than by a spreading inactivation, as previously thought.

Role of the *brahma* Gene and its Partners in Homeotic Gene Activation. As part of efforts to elucidate the molecular mechanisms governing cell fates in *Drosophila*, the roles of several of the previously identified genes have been studied. Several of these genes, including *brahma*, *osa*, *trithorax*, *moira*, and *verthandi*, were shown to be required for target gene transcription. The *brahma* gene encodes a large nuclear protein with homologs in yeast and man. In all organisms studied, the *brahma*-like proteins are part of large protein complexes that alter chromatin structure to facilitate gene expression.

GLOBAL NUTRITIONAL STRESS RESPONSES IN BACTERIA

The expression of the genomic repertoire of *Escherichia coli* is coordinately regulated in response to nutrient availability. Collectively, these responses determine growth as well as the ability to survive prolonged periods of starvation. The project conducted by ***Michael Cashel*** and his colleagues of the Section on Molecular Regulation focuses on the regulation of these processes in eubacteria by 3′ pyroesterified analogs of GDP and GTP that are abbreviated as (p)ppGpp. So far, these compounds have only been found in prokaryotes. Intracellular levels of (p)ppGpp respond to availability of amino acids, phosphate, nitrogen, or energy, as well as to other stress conditions; the ensuing (p)ppGpp signal alters global patterns of gene expression. In addition, (p)ppGpp can induce other global regulators and thereby recruit and integrate gene expression effects on broad genomic domains.



Robert Crouch (left) and Michael Cashel

Regulatory Mutants in RNA Polymerase Subunit Genes. This year, we studied isolates of mutants of RNA polymerase subunits that phenotypically mimic gene expression patterns shown by the presence of (p)ppGpp in strains genetically rendered devoid of (p)ppGpp. RNA polymerase holoenzymes reconstituted with either of two mutant sigma-70 subunits whose galP2 promoter is defective with respect to abortive RNA product formation, to promoter clearance, and to the stability of DNA enzyme open complexes. A total of 61 different mutant alleles of the RNA polymerase core subunit genes *rpoB* and *rpoC*, including 52 of our new isolates, have been screened and assigned to different functional classes with respect to phenotypes sensitive to (p)ppGpp, test promoters sensitive to (p)ppGpp, and functions regulated by other global regulators. These rankings will genetically define global regulatory pathways and lead to an understanding of these mechanisms.

Paralogous Genes Determine (p)ppGpp Synthesis and Degradation Functions. Eubacterial genomic sequencing has reinforced our view that the *relA* and *spoT* genes of *E. coli* are paralogous genes devoted to (p)ppGpp synthesis and breakdown, respectively. This implies that a duplication has occurred, followed by specialization favoring distinct functions. A single ancestral rel/spo gene encoding a protein with some degree of each of these specialized functions is widely distributed among prokaryotes, except in species closely related to *E. coli*. We have characterized one of these bifunctional ancestral proteins from a species of *Streptococcus*. The separate

protein domains for synthetic and degradation activities have been identified within protease-resistant cores, and their catalytic activities have been verified on protein fragments.

Polyphosphate Phosphatase and pppGpp Conversion to ppGpp. A second pair of *E. coli* gene paralogs exists that is also related to (p)ppGpp metabolism. The proteins of GppA and PpX are related, yet the *gppA* gene encodes the enzyme responsible for the major catalytic route of conversion of pppGpp to ppGpp, while the *ppx* gene encodes the enzyme that is the major source of polyphosphatase. Mutants of GppA lose only a small portion of polyphosphatase activity, mostly from *Ppx*. Conversely, although the Ppx protein can hydrolyze pppGpp *in vitro*, mutants of *ppx* do not display the phenotype of Gpp mutants: excessive accumulation of pppGpp compared with ppGpp. These apparently specialized activities are functionally related, since pppGpp is found to be a potent inhibitor of polyphosphatase activity. This can explain why stress conditions leading to pppGpp accumulation provoke polyP accumulation. The regulatory consequences of polyP accumulation are then coordinated with pppGpp regulation.

MOLECULAR GENETICS OF MAMMALIAN RETROVIRUS REPLICATION

The aim of the Section on Viral Gene Regulation, directed by ***Judith Levin***, is to define the molecular mechanisms involved in the replication of human immunodeficiency virus (HIV) and other mammalian retroviruses and in particular, to understand factors that influence the early phase of virus replication. This group has been using an *in vitro* approach to study events in reverse transcription and the role of the HIV nucleocapsid protein (NC) in increasing the efficiency and specificity of viral DNA synthesis.

Studies on Strand Transfer During HIV Replication. Two strand transfer events are required to complete elongation of minus- and plus-strand viral DNA and synthesis of the long terminal repeats at each end of proviral DNA. Levin and coworkers, in collaboration with L. Henderson and J. Bess (Frederick Cancer Research and Development Center), have shown that NC stimulates HIV-1 minus-strand transfer *in vitro*, in part by suppressing formation of self-priming products from (-) strong stop (SS) DNA. NC exerts this effect by destabilizing the large TAR secondary structure at the 3′ terminus of (-) SSDNA, thereby enabling (-) SSDNA to anneal to acceptor RNA. The biological significance of this previously unrecognized NC function is supported by the finding that, in endogenous reverse transcriptase (RT) assays with purified HIV-1 virions, self-priming products are not detected. In related work, Levin and colleagues have identified a drug that specifically targets minus-strand transfer in both *in vitro* and endogenous reactions. The potential importance of this drug for use in anti-HIV therapy is being investigated in experiments with HIV-1-infected T cells. Recent work in the laboratory has also led to the development of an *in vitro* system that reproduces events associated with plus-strand transfer: synthesis of (+) SSDNA; transfer to minus-strand acceptor DNA; and elongation of plus- and minus-strand DNAs to yield full-length double-stranded DNA.

Judith Levin

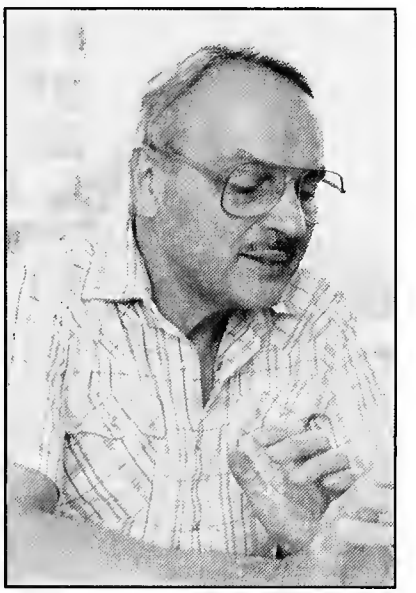
Mutational Analysis of Residues in Two Structurally-Defined Elements in HIV-1 RT. As shown by X-ray crystallography, the "primer grip" region in the p66 palm subdomain of HIV-1 RT contains residues that interact with bases at the 3′ end of the primer strand. This region is of interest, since Levin and colleagues previously showed that it is the 3′ bases of the polypurine tract plus-strand primer that are critical for initiation of plus-strand DNA synthesis. In collaboration with S. Le Grice (Case Western Reserve University), this group has now investigated the effect of alanine or aromatic substitutions in the primer grip residues. Almost all these RT mutants are defective in utilization of RNA plus- and minus-strand primers, but are able to extend DNA versions of these primers. Levin concludes that residues in the primer grip undergo unique interactions with RNA primers, which are qualitatively different from interactions with DNA primers, and may reflect specific recognition of the helical structure of a primer-template having RNA in the primer strand. In other studies with the RNA polypurine tract primer, Levin's group, in collaboration with S. Wilson and T. Kunkel (NIEHS), have been investigating the effect of alanine substitutions in residues in the "thumb"

subdomain, which also have specific contacts with the primer strand. These mutations affect RT positioning on the primer-template and lead to a loss of RNase H cleavage specificity. The results also show that proper RT positioning does not depend on the distance from the ends of the primer or template. These RT structure-function studies are beginning to illuminate the nature of protein-nucleic acid interactions with HIV-1 RT.

RECOMBINATION AND TRANSCRIPTIONAL CONTROL IN LAMBDOID PHAGES

Bacteriophage λ and its relatives have long been prime models for the study of basic molecular mechanisms. ***Robert Weisberg*** and his colleagues in the Section on Microbial Genetics have used this system to great advantage in their continuing studies on the control of gene expression and genetic recombination.

Antitermination of Transcription in Phage HK022. Transcription antitermination has long been studied as a major contributor to the control of gene expression in temperate bacteriophages, and has more recently been recognized as a control mechanism in many other organisms. Phage λ and its relatives, such as phage HK022, modify RNA polymerase so that it no longer responds to transcription terminators. Weisberg's group has shown that these two phages modify polymerase by different mechanisms. In HK022, specific sites found in unfinished phage transcripts directly interact with and change the elongation properties of transcribing RNA polymerase molecules so that they read through downstream terminators. In contrast to λ and other previously characterized systems, no protein factors are required. RNA polymerase mutations that specifically prevent HK022-directed polymerase modification have been identified and characterized. These mutations alter amino acids located in the zinc-binding region of the largest polymerase subunit, suggesting that this region of the enzyme recognizes the antitermination sites. Genetic and biochemical characterization of the antitermination sites shows that they consist of two hairpin stems that are separated by a single unpaired base. Some variation in the lengths of the stems and in the sizes and sequences of the terminal loops is compatible with function, and internal loops and bulges in one of the stems are important for activity. These results and others suggest that the largest RNA polymerase subunit participates in recognition of a specific RNA structure, and that this interaction prevents disruption of the elongating transcription complex when it arrives at termination sites.


Robert Weisberg

Site-Specific Recombination. Integrases are a family of enzymes that catalyze recombination between DNA molecules that carry specific sequences called attachment sites. Members of this family are required for efficient parasitism of bacterial cells by temperate bacteriophages. Weisberg's group has identified a subset of the amino acids and nucleotides that are responsible for the specificity of the protein-nucleic acid interaction in integrases encoded by phages λ and HK022. These two proteins are very closely related, but recognize different sequences within the core binding regions of their respective attachment sites. The determinants that are responsible for most of this difference in specificity consist of five amino acid differences in the two proteins and seven nucleotides in the two sets of attachment sites. It is likely that at least some of these amino acids and nucleotides interact directly with each other. This was shown by genetic studies, in which the effects of mutations in particular specificity-determining amino acids are precisely suppressed by mutations in particular specificity-determining nucleotides. Based on results obtained with these two integrases, Weisberg and his collaborators have proposed a general pathway for evolutionary changes in the specificity of protein-nucleic acid interactions.

RIBONUCLEASES H

Robert Crouch and his colleagues in the Section on Formation of RNA have continued their characterization of ribonucleases H. These enzymes are important for studying retroviral infections such as HIV, as well as being important therapeutic agents by means of antisense degradation of messenger RNA. In addition, these proteins occupy a unique niche in biochemical studies because of their ability to distinguish between RNA-DNA and

RNA-RNA duplexes and to degrade the RNA moiety of RNA-DNA hybrids. The details of this recognition and discrimination will advance our understanding of mechanisms by which proteins interact with nucleic acids.

RNases H of Bacteria Have Homologs in Eukaryotes. Crouch and his colleagues have shown that two types of RNases H found in bacteria also are present in eukaryotes. These are important findings in light of their potential involvement in antisense drug therapy and for assessment of the effects of inhibitors of HIV RNase H on cell functions resulting from inhibition of cellular RNases H.

Eukaryotes Have RNases H Similar to Bacterial RNase HI with an Additional Domain That Binds to Duplex RNAs. Crouch and his colleagues have also defined a non-RNase H domain present in one class of eukaryotic RNases H that binds to duplex RNAs, which might be involved in regulating RNase H activity. A common motif of 40 amino acids is present in all eukaryotic enzymes of this class, including yeasts and human cells. Interestingly, a protein involved in translating the polycistronic mRNA of cauliflower mosaic virus has the same motif and, when placed in the context of the yeast RNase H, functions as an RNA duplex binding site. This points to recognition of duplex RNAs by each of these proteins. This non-RNase H domain provides an opportunity to define the sites on the protein, in both domains, that bind to RNA-DNA hybrids and, thereby, significant insight into protein nucleic acid interactions. RNase H of this class purified to homogeneity has the undesirable property of binding to various surfaces, and can easily be lost. However, when duplex RNAs are included, the protein binds to them and remains soluble. This property can be exploited to determine the minimum size of duplex RNA necessary for binding and offers the potential for obtaining co-crystals of protein and nucleic acid for determining the structure by X-ray crystallography.

LABORATORY OF MOLECULAR GENETICS

Chief
Igor B. Dawid, Ph.D.

Senior Investigators
C. Michael Cashel, M.D., Ph.D.
Robert J. Crouch, Ph.D.
Judith G. Levin, Ph.D.
Thomas D. Sargent, Ph.D.
Robert A. Weisberg, Ph.D.

Tenure-Track Investigators
Susan Haynes, Ph.D.
James Kennison, Ph.D.
Brant Weinstein, Ph.D.
Ajay Chitnis, Ph.D.

Senior Fellows
Susana Cerritelli, Ph.D.
Jules Feledy, M.D.
Alexander Karavanov, Ph.D.
Maria Morasso, Ph.D.
Reiko Toyama, Ph.D.

Post-Doctoral Fellows
Margaret Adam, Ph.D.
Beth Agresta, Ph.D.
Arulvathani Arudchandran, Ph.D.
Katherine Ault, Ph.D.
Joseph Breen, Ph.D.
Jianhui Guo, M.D., Ph.D.
Li-ying Han, Ph.D.
Rodney King, Ph.D.
Laurent Kodjabachian, Ph.D.
Hongtao Ma, Ph.D.
Peter Madsen, Ph.D.
Undine Mechold, Ph.D.
Karen Neilson, Ph.D.
Catherine Nocente-McGrath, Ph.D.
Cecilia Pazman, Ph.D.
Michael Powell, Ph.D.
Michael Rebagliati, Ph.D.
Beth Roman, Ph.D.
Jean-Pierre Saint-Jeannet, Ph.D.
Jon Scales, Ph.D.
Samuel Stoler, Ph.D.
Michael Tsang, Ph.D.
Tiyun Wu, Ph.D.
Hirofumi Yamada, Ph.D.

Guest Researchers
Maureen Beanum, Ph.D.
Laszlo Dorgai, Ph.D.
Ya-Xiong Feng, Ph.D.
Tetsuhiro Kudoh, Ph.D.

Pre-Doctoral Fellows
Larissa Brown
Ting Yuan Chu
Michael Dedekian
Kevin Eggan
Christopher Reed
John Sandoval
Rosemary Wu
Erin Uyeda

Senior Technical Specialists
Helen Murphy
Klara Post
Martha Rebbert
Sieghild Sloan

ANNUAL BIBLIOGRAPHY

Agulnick AD, Taira M, Breen JJ, Tanaka T, Dawid IB, Westphal H. Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins. Nature 1996;384:270-2.

Banik-Maiti S, King RA, Weisberg RA. The antiterminator RNA of phage HK022. J Mol Biol 1997;272:677-87.

Bertuzzi S, Sheng HZ , Copeland NG, Gilbert DJ, Jenkins NA, Taira M, Dawid IB, Westphal H. Molecular cloning, structure, and chromosomal localization of the mouse LIM/homeobox gene *Lhx5*. Genomics 1996;36:234-9.

Brizuela BJ, Kennison JA. The *Drosophila* homeotic gene *moira* regulates expression of *engrailed* and HOM genes in imaginal tissues. Mech Dev 1997;65:209-20.

Chitnis A, Kintner C.Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. Development 1996;122:2295-301.

Dawid IB, Brown DD. Differentiation and gene regulation. Differentiation from bacteria to humans. Curr Opin Genet Dev 1996;6:523-5.

Fouquet B, Weinstein BM, Serluca FC, Fishman MC. Vessel patterning in the embryo of the zebrafish: guidance by axial mesoderm. Dev Biol 1997;183:37-48.

Ghosh M, Williams J, Powell MD, Levin JG, Le Grice SFJ. Mutating a conserved motif of the HIV-1 reverse transcriptase palm subdomain alters primer utilization. Biochemistry 1997;36:5758-68.

Guo J, Henderson LE, Bess J, Kane B, Levin JG. Human immunodeficiency virus type 1 nucleocapsid protein promotes efficient strand transfer and specific viral DNA synthesis by inhibiting TAR-dependent self-priming from minus-strand strong-stop DNA. J Virol 1997;71:5178-88.

Haruki M, Noguchi E, Kanaya S, Crouch RJ. Kinetic and stochiometric analysis of *Escherichia coli* ribonuclease HI binding to RNA-DNA hybrids measured by surface plasmon resonance. J Biol Chem 1997;272:22015-22.

Haynes SR, Cooper MT, Pype S, Stolow DT. Involvement of a tissue-specific RNA Recognition Motif protein in *Drosophila* spermatogenesis. Mol Cell Biol 1997;17:2708-15.

He X, Saint-Jeannet J-P, Wang Y, Nathans J, Dawid IB, Varmus H. A member of the frizzled protein family mediating axis induction by Wnt-5A. Science 1997;275:1652-4.

Heatwole VM, Haynes SR. Association of RB97D, an RRM protein required for male fertility, with a Y chromosome lampbrush loop in *Drosophila* spermatocytes. Chromosoma 1996;105:285-92.

Jen WC, Wettstein D, Turner D, Chitnis A, Kintner C. The Notch ligand, X-Delta-2, mediates segmentation of the paraxial mesoderm in Xenopus embryos. Development 1997;124:1169-78.

Katagiri C, Maeda R, Yamashika C, Mita K, Sargent TD, Yasumasu S. Molecular cloning of Xenopus hatching enzyme and its specific expression in hatching gland cells. Int J Dev Biol 1997;41:19-25.

King R A, Banik-Maiti S, Jin DJ, Weisberg R A. Transcripts that increase the processivity and elongation rate of RNA polymerase. Cell 1996;87:893-903.

Kuroda A, Murphy A, Cashel M, Kornberg A. Guanosine tetra- and pentaphosphate promoter accumulation of inorganic polyphosphate in Escherichia coli. J Biol Chem 1997;272:21240-3.

Minucci S, Leid M, Toyama R, Saint-Jeannet J-P, Peterson VJ, Horn V, Ishmael JE, Bhattacharyya N, Dey A, Dawid IB, Ozato K. Retinoid X receptor (RXR) within the RXR-retinoid acid receptor heterodimer binds it ligand and enhances retinoid-dependent gene expression. Mol Cell Biol 1997;17:644-55.

Morasso MI, Markova N, Sargent TD. Regulation of epidermal differentiation by a Distal-less homeodomain gene. J Cell Biol 1996;135:1879-87.

Morasso MI, Yonescu R, Griffin CA, Sargent TD. Localization of human DLX8 to chromosome 17q21.3-q22 by fluorescence in situ hybridization. Mamm Genome 1997;8:302-3.

Powell MD, Ghosh M, Jacques PS, Howard KJ, Le Grice SFJ, Levin JG. Alanine-scanning mutations in the "primer grip" of p66 HIV-1 reverse transcriptase result in selective loss of RNA priming activity. J Biol Chem 1997;272:13262-69.

Rebbert ML, Dawid IB. Transcriptional regulation of the *Xlim-1* gene by activin is mediated by an element in intron I. Proc Natl Acad Sci USA 1997;94:9717-22.

Schumann G, Cannon K, Ma W-P, Crouch RJ, Boeke JD. Antiretroviral effect of a gag-RNase HI fusion gene. Gene Therapy 1997;4:593-9.

Sheng HZ, Bertuzzi S, Chiang C, Shawlot W, Taira M, Dawid I, Westphal H. Expression of murine *Lhx5* suggests a role in specifying the forebrain. Develop Dynamics 1997;208:266-77.

Stainier DYR, Fouquet B, Chen J-N, Warren K, Weinstein BM, Meiler S, Pallithothingal M, Neuhauss SCF, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple D, Malicki J, Driever W, Fishman, M. C. Cardiovascular mutations in the zebrafish. Development 1996;123:285-92.

Taira M, Saint-Jeannet J-P, Dawid IB. Role of the *Xlim-1* and *Xbra* genes in anteroposterior patterning of neural tissue by the head and trunk organizer. Proc Natl Acad Sci USA 1997;94:895-900.

Toyama R, Dawid IB. *lim6*, a novel LIM homeobox gene in the zebrafish: comparison of its expression pattern with *lim1*. Dev Dynamics 1997;209:406-17.

Weinstein BM, Fishman MC. Cardiovascular morphogenesis in zebrafish. Cardiovasc Res 1996;31:E17-E24.

Weinstein BM, Schier AF, Abdelilah S, Malicki J, Solnica-Krezel L, Stemple DL, Stainier DYR, Zwartkruis F, Driever W, Fishman MC. Hematopoietic mutations in the zebrafish. Development 1996;123:303-9.

Winning RS, Scales JB, Sargent TD. Disruption of cell adhesion in Xenopus embryos by kinase activity of Pagliaccio, and Eph-class receptor tyrosine kinase. Dev Biol 1996;179:309-19.

Wu W, Henderson LE, Copeland TD, Gorelick RJ, Bosche WJ, Rein A, Levin JG. Human immunodeficiency virus type 1 nucleocapsid protein reduces reverse transcriptase pausing at a secondary structure near the murine leukemia virus polypurine tract. J Virol 1996;70:7132-42.

Zhan X, Crouch RJ. The isolated RNase H domain of murine leukemia virus reverse transcriptase: retention of activity with concomitant loss of specificity. J Biol Chem 1997;272:22023-9.

LABORATORY OF MOLECULAR GROWTH REGULATION

Bruce H. Howard, M.D., Chief

The Laboratory of Molecular Growth Regulation conducts research on the control of cell proliferation, gene regulation, and DNA replication in eukaryotic cells. In the area of gene regulation, independent groups work in complementary areas, including gene regulation of the developing immune system, control of gene expression during early embryogenesis, gene silencing mediated by epigenetically inherited higher order chromatin domains, RNA polymerase III-dependent transcription of small RNA-encoding genes, and the mechanisms by which gene-specific transcriptional activators interact with the basal RNA polymerase II transcriptional machinery. A collaborative atmosphere is fostered in this laboratory by the overlapping interests of its investigators, as well as by an active schedule of seminars and group meetings.

DEVELOPMENTAL GENE REGULATION OF THE IMMUNE SYSTEM

The Section on Molecular Genetics of Immunity, led by ***Keiko Ozato***, investigates gene regulation in the immune system. Within this framework, this group analyzes transcription of MHC class I and other genes that are regulated by retinoids and interferons. The primary recent focus has been to elucidate the roles of two transcription factors, RXRβ and interferon consensus sequence binding protein (ICSBP). RXRβ is a member of the nuclear hormone receptor superfamily which, together with retinoic acid receptor (RAR), is involved in retinoid-mediated transcription. ICSBP is a member of the interferon regulatory factor (IRF) family and plays a role in gene regulation mediated by interferons and viruses.

Keiko Ozato

Ligand-Bound RXR/RAR Heterodimer Recruits the Histone Acetylase PCAF. Unliganded nuclear hormone receptor heterodimers such as RXR/RAR are thought to repress transcription by associating with a histone deacetylase-corepressor complex. Conversely, ligand-induced transcriptional activation by the heterodimer may require local histone acetylation that could alter the repressive chromatin environment. We discovered that recombinant RXR/RAR heterodimers complexed with cognate target DNA elements recruit the histone acetylase PCAF in human cell nuclear extracts, and that this occurs only upon retinoid addition. As a result, histone acetylase activity accumulates on the heterodimer-DNA complex in a ligand-dependent manner. *In vitro* studies have shown that recombinant PCAF binds to the heterodimer following ligand-induced release of a co-repressor (N-CoR/RIP13), and without requiring p300/CBP, which can bind independently to the heterodimer. Domain analysis revealed that the C-terminal domain of PCAF that is homologous to yeast GCN5 interacts with the receptors mainly through their DNA binding domains. Finally, we found that transfection of PCAF into NIH3T3 cells leads to enhanced retinoid-response reporter activity, supporting a role for PCAF in ligand-dependent transcription. Together, these results indicate that, upon ligand binding, the heterodimer becomes associated with a histone acetylase, presumably in exchange with release of a histone deacetylase-corepressor complex, thereby generating a transcriptionally active chromatin environment.

A Histone Deacetylase Inhibitor Potentiates Retinoid Receptor Action in Embryonal Carcinoma Cells. To gain further insight into the role of histone acetylation in retinoid-dependent transcription, we studied the effects of trichostatin A (TSA), a specific inhibitor of histone deacetylase, on P19 embryonal carcinoma cells. We showed that co-addition of TSA and RA markedly enhances neuronal differentiation in these cells, although TSA alone does not induce differentiation, but causes extensive apoptosis. Consistent with the cooperative effect of TSA and RA, co-addition of the two agents was found to synergistically enhance transcription from stably integrated RA responsive promoters. The transcriptional synergy by TSA and RA requires the RA responsive element

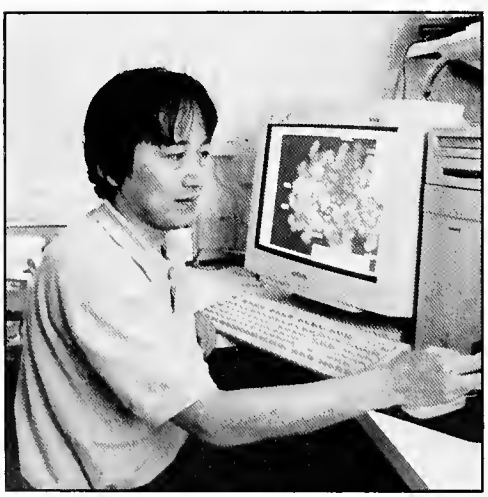
(RARE) and a functional RXR/RAR heterodimer, both obligatory for RA-dependent transcription. Furthermore, we found that TSA causes promoter activation by an RXR-selective ligand that is otherwise inactive in transcription. Finally, these studies revealed that TSA alone or in combination with RA increases *in vivo* endonuclease sensitivity within the RA responsive promoter, suggesting that TSA treatment might alter a local chromatin environment to enhance RXR/RAR heterodimer action. Taken together, these results indicate that histone acetylation influences activity of the heterodimer, which is in line with the observed interaction between the RXR/RAR heterodimer and a histone acetylase.

ICSBP-Deficient Mice Display Impaired Resistance to Intracellular Infection Due to a Primary Defect in IL-12p40 Induction. Mice lacking the transcription factor ICSBP rapidly succumb to fulminant infection with the intracellular protozoan *Toxoplasma gondii*, a defect associated with a loss in IL-12p40 and IFNg responses to the parasite. In contrast, effector functions downstream from the IL-12/IFNg deficiency are intact in the ICSBP -/- animals. Further analysis *in vitro* revealed that macrophages from uninfected ICSBP -/- mice are selectively impaired in their ability to express IL-12p40, but not other cytokines, in response to conventional stimuli. These data demonstrate that ICSBP plays a critical role in IL-12p40 gene activation and thus in regulating IFNg-dependent immunity.

EUKARYOTIC TRANSCRIPTIONAL REGULATION

The Section on Eukaryotic Transcriptional Regulation, led by ***Yoshihiro Nakatani***, endeavors to understand the mechanisms by which: (1) gene-specific transcriptional activators interact with the RNA polymerase II transcription machinery to regulate messenger RNA synthesis; and (2) tumor viral proteins perturb normal cellular interactions for transforming cells.

Histone Octamer-Like Substructure in TFIID. To probe the architecture of the TFIID complex, in collaboration with Alan Hinnebusch (Laboratory of Eukaryotic Gene Regulation), we have employed yeast suppressor screening. Several temperature-sensitive mutants were isolated by introducing mutations in the histone-like region of yeast TAF60 (yTAF60), a homolog of dTAF62. One of these mutants was used to identify extragenic suppressors from a multicopy plasmid bank. A histone-H3-like TAF (yTAF20), a histone-H2B-like TAF (yTAF61), and yTAF90, which has WD40-repeats, were isolated as suppressors. Moreover, biochemical studies with human homologs indicate that histone-H2B-like TAF forms a homomeric complex, which interacts with the histone-H3/H4-like TAFs. These results suggest that TFIID contains a histone-octamer-like structure composed of two dimers of the histone-H2B-like TAF attached to a tetramer of histones-H3/H4-like TAFs.


Yoshihiro Nakatani

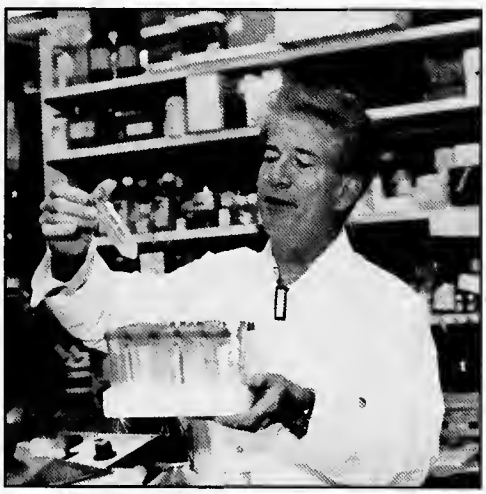
Histone Acetylase Activities in Multiple Transcription Factors. In previous work, we cloned PCAF, a factor that competes with the viral oncogene E1A for binding to the closely related transcriptional coactivators p300 and CBP. Based on its homology to yeast GCN5 and the fact that yGCN5 is a histone acetylase, intrinsic histone acetyltransferase activity in PCAF was identified. PCAF acetylates histones H3 and H4, but preferentially H3 in both free histones and the mononucleosome.

Only recently has it been appreciated how many sequence-specific DNA-binding proteins bind to the coactivators p300 and CBP (often referred to as p300/CBP to signify their closely related functions). Indeed, p300/CBP is a large protein consisting of over 2,400 amino acids, known to interact with nuclear hormone receptors, CREB, c-Jun, c-Myb, Sap-1a, c-Fos and MyoD. Furthermore, p300/CBP recruits coactivators PCAF and ACTR to activate transcription. Over the past year, we and our collaborators demonstrated that PCAF, p300, CBP, ACTR, and TAFII250 all have intrinsic histone acetylase activity. These findings suggest that targeted histone acetylation at specific promoters may be a very general mechanism by which RNA polymerase II transcriptional machinery gains access to transcriptionally repressed chromatin.

INITIATION OF DNA REPLICATION IN MAMMALIAN CHROMOSOMES

Central to understanding how eukaryotic cells and the viruses that proliferate within them regulate the replication of their genomes is the nature of both DNA sites where replication begins and the proteins that interact with them. Intriguingly, while DNA alone contains many potential initiation sites for its replication, differentiated cells in higher eukaryotes select specific sites along their chromosomes to initiate DNA replication. The Section on Eukaryotic DNA Replication, led by ***Mel DePamphilis***, focuses on these problems, as well as on how the processes of DNA replication and transcription are turned on at the beginning of mammalian development.

The DHFR Gene Initiation Zone for DNA Replication Contains at Least Two and Probably Three Primary Initiation Sites. Analyses of mammalian DNA replication origins by different methods have led to contradictory conclusions. Mapping the origins of nascent DNA strands labeled during their biosynthesis suggests that initiation occurs at specific origins of bidirectional replication (OBR) of 0.5 to 2 kb, whereas mapping the origins of replication bubble and fork structures by 2D gel electrophoresis suggests that initiation events are distributed almost uniformly throughout initiation zones as large as 55 kb. To determine whether or not initiation zones consist of one or more primary initiation sites, nascent DNA was labeled with bromodeoxyribouridine (BrdU) in synchronized CHO cells as they entered S-phase, and the ratios of nascent DNA strands to non-replicated DNA were measured quantitatively, using competitive PCR at 26 different sequences in a 140 kb region that contained the 55 kb DHFR (dihydrofolate reductase) gene initiation zone. The results demonstrated that the 12 kb region exhibiting the greatest frequency of replication bubbles in 2D gels contains two primary initiation sites for DNA replication. A sharp peak of BrdU-labeled nascent DNA occurs at the ori-β OBR (~17 kb downstream of the DHFR gene), where initiation events are 12 times more frequent than at distal sequences, in excellent agreement with several previous studies using either synchronized or unsynchronized cells. A second, previously unrecognized, initiation site (ori-β′) was detected 5.5 kb further downstream. These two replication origins, together with the previously identified ori-γ about 23 kb downstream of ori-β are the sites where initiation events are most frequent in the DHFR initiation zone.


Mel DePamphilis

Specific Initiation Sites for DNA Replication First Appear in Late G1-Phase Nuclei. Previous studies have shown that *Xenopus* egg extract can initiate DNA replication in purified DNA molecules, but only after the DNA becomes organized into a pseudo-nucleus. However, DNA replication under these conditions is independent of DNA sequence and begins at many sites distributed randomly throughout the molecules, whereas DNA replication in the chromosomes of mammalian cells initiates at specific, heritable sites. Our recent studies have shown that *Xenopus* egg extract can initiate DNA replication at specific sites in mammalian chromosomes, but only when the DNA is presented in the form of an intact nucleus isolated from mammalian cells. Initiation under these conditions occurs at or close to the same sites (e.g., ori-β) used by the host cell. Subsequent studies by others revealed that specific initiation sites do not appear in mammalian nuclei until late G1-phase. Together, these results suggest that initiation sites for DNA replication in mammalian cells are established during late G1-phase by some component of nuclear structure, and that these sites are then activated by soluble factors present at the beginning of S-phase.

Confirming and extending these observations, we have shown that the amount of DNA synthesis observed in early G1-nuclei and the ability to initiate specifically at the ori-β OBR depend on experimental conditions. In particular, we have found conditions in which incubation of late G1-nuclei in *Xenopus* egg extract yields a very sharp peak of nascent DNA at ori-β OBR and a small peak at ori-β′, in excellent agreement with mapping studies carried out on nascent DNA synthesized in hamster cells. Therefore, we can now activate *in vitro* the same pre-replication complexes established *in vivo* by hamster cells during late G1-phase using soluble *Xenopus* egg factors. Moreover, we have found that the extent of replication observed in early G1-nuclei, but not in late

G1-nuclei, depends on one or more replication factors provided by the *Xenopus* egg extract. This system should prove extremely useful for exploring the assembly of site-specific DNA replication complexes in mammalian nuclei.

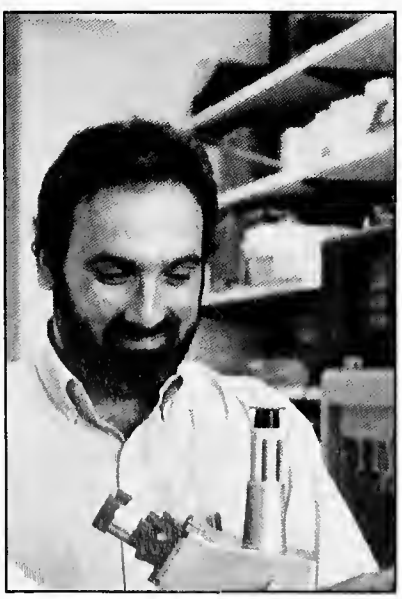
Identification of Proteins Required for Site-Specific Initiation. Initiation of yeast DNA replication depends on an origin recognition complex (ORC) of six proteins that bind to yeast replication origins, on cdc6 that then binds to ORC, and finally on minichromosome maintenance (MCM) proteins that then bind to ORC/cdc6 complexes. Recent studies in other laboratories have shown: (1) that mammals and other higher eukaryotes contain homologs of these yeast proteins; and (2) that immunodepletion of these proteins from *Xenopus* egg extracts eliminates their ability to initiate DNA replication on DNA or on sperm chromatin added to the extract. Therefore, it appears that yeast is a valid paradigm for initiation of DNA replication in higher eukaryotes.

In an effort to identify proteins associated with specific replication origins in mammalian chromosomes, we have cloned and sequenced the ORC1 and ORC2 homologs from Chinese hamsters, and demonstrated that antibodies raised against peptides derived from these proteins will immunoprecipitate ORC1 or ORC2 expressed *in vitro*. In addition, these proteins have been tagged at their N-terminus with unique polypeptides that can be recognized by specific monoclonal antibodies. We are now in the process of developing methods to recover specific DNA sequences to which the ORC binds and thereby to determine whether or not ORC is bound specifically at ori-β and ori-β′ in late G1-phase CHO cells. We also have specific antibodies directed against MCM3. This protein, which is thought to be bound specifically to pre-replication complexes, was found bound to chromatin throughout G1-phase, so it will be of considerable interest to determine whether or not it is associated specifically with ori-β and ori-β′ in late G1-phase CHO cells.

MOLECULAR REGULATION OF GENE EXPRESSION

RNA polymerase (pol) III is responsible for synthesizing tRNAs, 5S rRNA, U6 snRNA, as well as additional small transcripts such as Alu RNA and Y RNAs. The Unit on Molecular and Cell Biology, led by ***Richard Maraia***, seeks to understand the mechanisms that regulate expression of these RNAs.

Mechanisms of RNA Polymerase III Transcription. Although tRNA and 5S rRNA are essential transcripts that play central roles in protein synthesis and gene expression, understanding of their regulation is largely limited to the assembly of transcription complexes on their genes. Since pol III transcription factors remain stably associated with their target promoters through multiple rounds of RNA synthesis, any regulation of transcription would have to occur either through repeated disassembly and assembly of the complex, or perhaps by the less-explored alternative, the controlled use of the pre-assembled complexes. Indeed, recent advances indicate that human La protein can control pol III transcription complexes and that this activity can be regulated by phosphorylation and dephosphorylation.


Richard Maraia

Human La antigen is a 408-amino-acid phosphoprotein, which facilitates transcription termination and reinitiation by pol III. La is found associated with precursor tRNAs and other nascent transcripts synthesized by pol III, binding to the oligo(U) tract common to all such nascent transcripts. By binding to this RNA motif, La protects the 3′ ends of newly released nascent transcripts so that proper 3′-end maturation can occur. Additionally, La can control stable transcription complexes independent of their assembly. Free La protein, but not La protein bound to RNA, is active in transcription, suggesting a mechanism by which feedback-inhibition of RNA synthesis can be mediated by La. According to this model, La activates transcription only when it is not associated with nascent RNA. As the nascent transcript dissociates from La to participate in its cellular function, La is converted to a free form that can activate another round of RNA synthesis. Thus, a need for RNA may be sensed by the cell through La.

Phosphorylation and Dephosphorylation Regulate La Activity. In a novel chromatographic purification scheme, we separated native La isolated from HeLa cells into two distinct forms, one containing a single

phosphate on serine-366 and the other unphosphorylated. Phosphoserine-366 resides within a CKII consensus target site next to the basic region of La that is required for transcription factor activity. Both the C-terminal basic region of La and the adjacent CKII target site have been conserved in La proteins found in species ranging from yeast to man.

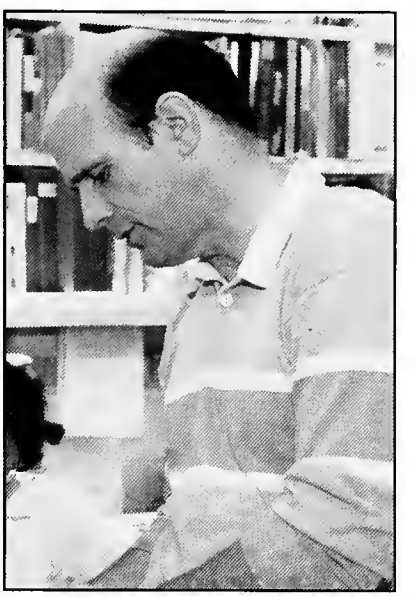
While both phosphorylated and unphosphorylated forms of native La bind to RNA, we found that only the unphosphorylated form is active for transcription. We demonstrated that activation of phospho-La can be achieved by enzymatic removal of phosphate at residue 366. This result, coupled with earlier data showing that the phosphate in La is turned over much more rapidly than La protein, along with evolutionary conservation of the CKII consensus site in the conserved C-terminus of La, provides strong evidence that CKII-mediated phosphorylation and subsequent dephosphorylation control La activity *in vivo*.

A Novel Regulatory Mechanism Discovered Using the Pol III Model of Eukaryotic Transcription: Controlled Recycling of Transcription Complexes. Although assembly of the pol III complex is the rate-limiting step for *de novo* transcription initiation, once formed, these complexes are stable and undergo multiple reinitiations by pol III. Previously characterized examples of class III gene regulation involve modulating the amount or activity of transcription complex assembly factors. By contrast, La controls recycling of the stable transcription complex. Moreover, it appears likely that factors other than La will prove to be involved in modulating recycling efficiency, e.g., pol III-associated transcription factors and/or pol III itself, through which La presumably works.

CONTROL OF MAMMALIAN CELL PROLIFERATION

The Human Genetics Section, under the direction of ***Bruce Howard***, investigates mammalian cell growth regulation, with particular attention to mechanisms underlying cellular immortalization and its converse, cellular senescence.

Histone Acetyltransferases. The growth-regulatory properties of the histone acetyltransferase PCAF were examined in much greater detail than previously. Contrary to transient expression results obtained with HeLa cells, log phase growth of several cell types, including normal human fibroblasts, Saos2 osteosarcoma cells, and HeLa cells, was found to be normal or near normal following stable transduction with a PCAF-encoding retrovirus. These results imply that PCAF, when overexpressed, does not act as a non-specific inhibitor of cell proliferation, but rather is involved as an effector in terminal differentiation and potentially in other pathways that lead to growth arrest. Elevated expression levels of PCAF in conjunction with terminal differentiation and senescence are consistent with this interpretation.



Bruce Howard

To determine to what extent the histone acetyltransferase (HAT) activity of PCAF influences the cell cycle exit associated with cell senescence, wild-type or HAT-defective forms of PCAF were overexpressed in non-immortalized fibroblasts. HAT-defective PCAF was generated by introducing alanine-scanning point mutations into the putative acetyl-CoA binding domain of this protein. Three such mutations, involving single or double amino acid changes, were shown to eliminate virtually all HAT activity in *Baculovirus*-produced affinity-purified recombinant protein. Results of these studies to date indicate that PCAF is indeed an effector in senescence-related cell cycle exit, but also that its actions are likely to be complex.

The viral oncoprotein E1A competes with PCAF for binding to p300/CBP. Because E1A possesses transcription activation as well as repression activity depending on promoter context, it was considered that E1A may recruit a HAT other than PCAF in some instances. In collaboration with the Nakatani group, we showed this to be the case, with p300/CBP itself proving to be the sought-after HAT. The HAT domain of p300/CBP was mapped to a central region between the bromodomain and the PCAF interaction site. Several collaborations are underway with the Nakatani and other groups to assess the requirement for p300/CBP HAT activity in transcriptional activation at various promoters.

Histone Deacetylases. The first mammalian histone deacetylase (HD) identified, hHD1, was found to be a homolog of the yeast pleiotropic transcription factor RPD3. Previously, we cloned a second human histone deacetylase encoding gene, hHD2. Over a 300 amino acid N-terminal conserved region, hHD2 shares 66% identity at the amino acid level with hHD1. hHD1 and hHD2 proteins, when overproduced in a *Baculovirus* expression system and affinity-purified, possess HD activity using histone H4 as a substrate. However, in each case activity is too low to account for that detected in crude cellular extracts, suggesting that additional HDs remain to be identified. Consistent with this possibility, none of the known RPD3-homologous HDs (hHD1, hHD2, or mRPD3) copurifies with the major HD activity in HeLa cells, which was found to migrate as a ≈300 kDa complex.

Two HD families have been reported to exist in *Saccharomyces cerevisiae*, represented by yRPD3 and by yHDA1, respectively. In additional work on mammalian HDs, a human gene related to the yHDA1 protein was detected in the human EST database, and clones covering the full length cDNA were obtained. The predicted protein, 124 kDa, shares homology with yHDA1 over a 350 amino acid region near its C-terminus, and contains highly conserved amino acid residues found in both RPD3-like HDA1-like families, but otherwise shares no similarities with proteins in the public databases.

LABORATORY OF MOLECULAR GROWTH REGULATION

Chief
Bruce H. Howard, M.D.

Principal Investigators
Melvin L. DePamphilis, Ph.D.
Yoshihiro Nakatani, Ph.D.
Keiko Ozato, Ph.D.

Tenure-Track Investigator
Richard J. Maraia, M.D.

Senior Fellows
Dau Yin Chang, Ph.D.
Anup Dey, Ph.D.
Hao Fan, Ph.D.
Glen Humphrey, Ph.D.
Kotaro Kaneko, Ph.D.
Vasily Ogryzko, Ph.D.
Valya Russanova, Ph.D.
Xiang-Jiao Yang, Ph.D.

Post-Doctoral Fellows
Nissan Battacharya, Ph.D.
Jorge Blanco, Ph.D.
Joseph Bogan, Ph.D.
Jae Hun Cheong, Ph.D.
Cristina Contursi, Ph.D.
John Goodier, Ph.D.
Laura Augeri Henmueller, Ph.D.
Chun Hong, M.D.
Ying Huang, Ph.D.
Takehiko Kobayashi, Ph.D.
Takeshi Kuwata, M.D., Ph.D.
Bruno LeFebvre, Ph.D.
Congiun Li, Ph.D.
Jiaming Lu, Ph.D.
Atsuko Masumi, Ph.D.
Darren Natale, Ph.D.
Theo Rein, Ph.D.
Shigekazu Sasaki, Ph.D.
R. Louis Schiltz, Ph.D.
Mark Swanson, Ph.D.
Alex Vassilev, Ph.D.
I-Ming Wang, Ph.D.
Wang Yonghong, Ph.D.

Guest Researchers
Levra Balsam
Valerie Horn, Ph.D.
Tetsuo Maruyama, Ph.D.
Giulia Paggio, Ph.D.

Pre-Doctoral Fellows
Farideh Chitsaz
Anita Chu
James Elmer
Amini Farhang
Erik Pierstorff

ANNUAL BIBLIOGRAPHY

Bhattacharyya N, Dey A, Minucci S, Zimmer A, John S, Hager G, Ozato K. Retinoid induced chromatin structure alterations in the RARß2 promoter. Mol Cel Biol 1997;17:6481-90.

Chang DY, Newitt J, Hsu K, Bernstein H, Maraia RJ. A highly conserved nucleotide in the Alu domain of SRP RNA mediates SRP function through high affinity binding to SRP9/14. Nucl Acids Res 1997;25:1117-22.

Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM. Nuclear receptor co-activator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 1997;90:569-80.

Dey A, Ozato K. Genomic footprinting of retinoic acid regulated promoters in embryonal carcinoma cells. Methods (Academic press). Ed: Zaret, K. 1997;11:197-204.

Englander EW, Howard BH. Alu-mediated detection of DNA damage in the human genome. Mutat Res 1997;385:31-9.

Fan H, Sakulich AL, Goodier JL, Zhang H, Qin J, Maraia RJ. Phosphorylation of the human La antigen on serine 366 can regulate recycling of RNA polymerase III transcription complexes. Cell 1997;88:707-15.

Goodier JL, Fan H, Maraia RJ. A carboxy-terminal basic region controls the RNA polymerase III transcription factor activity of human La protein. Mol Cell Biol 1997;17:5823-32.

Herrera JE, Bergel M, Yang X-J, Nakatani Y, Bustin M. The histone acetyltransferase activity of human GCN5 and PCAF is stabilized by coenzymes. J Biol Chem 1997;272:27253-8.

Humphrey GW, Englander EW, Howard BH. Specific binding sites for a pol III transcriptional repressor and pol II transcription factor YY1 within the internucleosomal spacer region in primate Alu repetitive elements. Gene Expr 1996;6:151-68.

Imhof A, Yang X-J, Ogryzko VV, Nakatani Y, Wolffe AP, Ge H. Acetylation of general transcription factors by histone acetylases: identification of a major site of acetylation in TFIIEβ. Curr Biol 1997;7:689-92.

Jurutka PW, Hsieh JC, Remus LS, Whitfield GK, Thompson PD, Haussler CA, Blanco JC, Ozato K, Haussler MR. Mutations in the 1,25-dihydroxyvitamin D3 receptor identifying C-terminal amino acids required for transcriptional activation that are functionally dissociated from hormone binding, heterodimeric DNA binding, and interaction with basal transcription factor IIB, in vitro. J Biol Chem 1997;272:14592-9.

Kaneko KJ, Cullinan EB, Latham K E, DePamphilis ML. Transcription factor mTEAD-2 is selectively expressed at the beginning of zygotic gene expression in the mouse. Development 1997;124:1963-73.

Kushida MM, Dey A, Zhang XL, Campbell J, Heeney M, Carlyle J, Ganguly S, Ozato K, Vasavada H, Chamberlain JW. A 150-base pair 5' region of the MHC class I HLA-B7 gene is sufficient to direct tissue-specific expression and locus control region activity: the alpha site determines efficient expression and in vivo occupancy at multiple cis-active sites throughout this region. J Immunol 1997;159:4913-29.

Majumder S, Zhao Z, Kaneko K, DePamphilis ML. Developmental Acquisition of Enhancer Function Requires a Unique Co-activator Activity. EMBO J 1997;16:1721-31.

Martin BK, Chin C-H, Olsen JC, Dey A, Ozato K, Ting J-Y. Induction of MHC class I expression by the MHC class II transactivator CIITA. Immunity 1997;6:591-600.

Minucci S, Horn V, Bhattacharyya N, Russanova V, Ogryzko VV, Gabriele L, Howard BH, Ozato K. A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. Proc Nat Acad Sci USA 1997;94:11295-300.

Minucci S, Leid M, Toyama R, Sant-Jeannet J-P, Peterson VJ, Horn V, Bhattacharyya N, Dey A, Dawid IB, Ozato K. Retinoid X receptor (RXR) within the RXR/RAR heterodimer binds its ligand and enhances retinoid-dependent gene expression. Mol Cell Biol 1997;17:644-55.

Minucci S, Ozato K. Retinoid receptors in transcription. Curr Op Genet Dev 1996; 6:567-74.

Mizzen C A, Yang X J, Kokubo T, Brownell JE, Bannister AJ, Owen-Hughes, Workman J, Berger SL, Kouzarides T, Nakatani Y, Allis CD. The $TAD_{II}250$ subunit of TFIID has histone acetyltransferase activity. Cell 1996;87:1261-70.

Nishikawa J, Kokubo K, Horikoshi M, Roeder RG, Nakatani Y. Drosophila $TAF_{II}230$ and the transcriptional activator VP16 bind competitively to the TATA box-binding domain of TBP. Proc Natl Acad Sci USA 1997;94:85-90.

Nunez SB, Medin JA, Braissant O, Kemp L, Wahli W, Ozato K, Segars JH. Retinoid X receptor and peroxisome proliferator-activated receptor activate an estrogen responsive gene independent of the estrogen receptor. Mol Cell Endocrinol 1997;127:27-40.

Ogryzko VV, Brinkmann E, Howard BH, Pastan I, Brinkmann U. Antisense inhibition of CAS, the human homologue of the yeast chromosome segregation gene CSE1, interferes with mitosis in HeLa cells. Biochemistry 1997;36:9493-500.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 1996;87:953-9.

Ogryzko VV, Wong P, Howard BH. WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. Mol Cell Biol 1997;17:4877-82.

Puri PL, Sartorelli V, Yang X-J, Hamamori Y, Ogryzko VV, Howard BH, Kedes L, Wang JYJ, Graessmann A, Nakatani Y, Levrero M. Differential roles of p300 and PCAF acetyltransferases in muscle differentiation. Mol Cell 1997;1;35-45.

Rein T, Natale DA, Gärtner U, Niggemann M, DePamphilis ML, Zorbas H. Absence of an unusual "densely methylated island" at the hamster DHFR Ori-β. J Biol Chem 1997;272:10021-9.

Rein T, Zorbas H, DePamphilis ML. Active mammalian replication origins are associated with a high density cluster of ^{m}CpG dinucleotides. Mol Cell Biol 1997;17:416-26.

Samuel CE, Ozato K. Induction of interferon and interferon-induced genes. In: Pestka S, Schellekens H, eds. Biotherapy. Cytokine Yearbook, vol 8. Dordrecht, the Netherlands: Kluwer Academic Publisher, 1996;183-7.

Sarrowa J, Chang DY, Maraia RJ. The decline in human Alu transposition was accompanied by an asymmetric decrease in SRP9/14 binding to dimeric Alu RNA and increased expression of small cytoplasmic Alu RNA. Mol Cell Biol 1997;17:1144-51.

Sharf R, Meraro D, Aziel A, Thornton A, Ozato K, Petricoin EF, Larner AC, Schaper F, Hauser H, Levi B-Z. Phosphorylation of ICSBP modulates DNA binding and interactions with other IRF proteins. J Biol Chem 1997;272:9785-92.

Scharton-Kersten T, Contursi C, Masumi A, Sher A, Ozato K. ICSBP-deficient mice display impaired resistance to intracellular infection due to a primary defect in IL-12 p40 induction. J Exp Med 1997;186:1523-34.

Sunaga A, Maki K, Lagasse E, Blanco J, Ozato K, Miyazaki J, Ikuta K. Myeloid differentiation is impaired in transgenic mice with targeted expression of a dominant negative form of Retinoid X Receptor ß. Br J Haematol 1997;96:19-30.

Thornton AM, Ogryzko VV, Dent A, Sharf R, Levi BZ, Kanno Y, Staudt LM, Howard BH, Ozato K. A dominant negative mutant of an IFN regulatory factor family protein inhibits both type I and type II IFN-stimulated gene expression and antiproliferative activity of IFNs. J Immunol 1996;157:5145-54.

Wiekowski M, Miranda M, Nothias J-Y, DePamphilis ML. Changes in histone synthesis and modification at the beginning of mouse development correlate with the establishment of chromatin mediated repression of transcription. J Cell Science 1997;110:1147-58.

LABORATORY OF PHYSICAL AND STRUCTURAL BIOLOGY

V. Adrian Parsegian, Ph.D., Chief

Motivated to understand biological structures through the physical forces that animate them, the first theme of this Laboratory's work is to examine forces between and within biological macromolecules. To this end, we measure force vs. separation between molecules from all classes of bio-matter: lipids, proteins, nucleic acids, and lipids. The same "osmotic stress" strategy used for forces between molecules allows us to move the parts of single molecules to change the conformation of molecules such as ionic channels and hemoglobin. Our second theme concerns the response of single ionic channels to the stress of solutes, the dynamics of molecular movement by channel molecules and by the particles that pass through them. A third theme is to observe the formation of ordered molecular assemblies ("liquid-crystals") of molecules such as DNA or lipids whose energies of assembly can be measured and used to understand the connection between molecular force and organization. Our "alchemy" combines molecular biologists, physical biochemists, experimental and theoretical physicists. The mix creates a practical science, a new logic for thinking about molecular recognition, conformation, and assembly.

MOLECULAR INTERACTION AND ORGANIZATION

The Section on Molecular Biophysics, directed by ***Adrian Parsegian***, studies the organization of molecular assemblies. Its current interests include: DNA/lipid assemblies, of major interest for improved transfection; DNA/protein complexes, measuring the strength, specificity, longevity, and hydration of these complexes as a function of mutations in DNA in order to probe the connection between these properties of association and DNA expression; DNA assemblies such as are seen in viral capsids and *in vitro*, to ascertain DNA pressure and to watch the patterns of association of these "semi-flexible" polymers; lipid/water systems whose malleability creates elaborate "phase diagrams" and whose work of deformation and transformation can now be measured in order to devise systematic predictions of how membranes are driven to change shape; telomeric DNA analogs, polypeptides, and polysaccharides, whose intermolecular forces will allow us to build a full picture of how biological molecules do their work. A significant goal is to characterize and codify measured forces in such a way as to be used in computation. The Section also works on fundamental problems such as the "vapor pressure paradox", a 50-year-old puzzle as to why lipids imbibe less water from a vapor than from a solution with the same activity of water. We are applying our recent theory of this paradox to the question of when a the solution properties of large biological molecules can be applied to behavior within the confines of a virus or cell. Finally, under a NASA/NIH Inter Agency Agreement, we are examining new lenses for x-rays.

DNA-Lipid Assemblies: Gene Therapy Using Cationic Lipid/DNA Complexes. One of the problems in gene therapy is how to deliver DNA into target cells in the body. Delivery systems have to overcome two obstacles: natural defense mechanisms against foreign DNA in the blood stream and delivery through cell membranes. Complexes made from cationic lipids (e.g., TAP, DAB) and DNA seem to work, but so far the *in vivo* activity is not yet sufficient to apply these systems to humans. (Cationic lipids are also highly toxic.) In collaboration with a group at the National Cancer Institute, we are performing an x-ray diffraction study on complexes with different lipids in order to relate their *in vivo* activity to their structural and packing properties. It appears that the most active complexes have lamellar structure, in which layers of parallel DNA molecules are sandwiched between lipid bilayers. These complexes are not only of great practical interest, but they are also a useful model system of polyelectrolyte adsorption on charged surfaces.

DNA-Protein Complexes. Osmotic stress experiments on DNA-protein complexes are our first attempt to bridge the gap between force measurements and binding reactions. The correct functioning of transcription factors and other regulatory proteins that recognize specific DNA sequences requires not only binding with high affinity to the proper sequence but also the ability to distinguish effectively the recognition sequence from all others, including those that differ by only one or two base pairs. A key, fundamental problem in biophysics is understanding how such binding strength and specificity are so tightly linked in recognition reactions. We are focusing on the role of water in this process. The correlation between numbers of sequestered water molecules and binding free energies as surfaces are mutated or as water is removed at high stresses is directly pertinent

to uncovering a link between recognition and hydration. On a practical level, the osmotic stress dependence of the energetics of DNA-protein complexes offers novel possibilities for correlating thermodynamics and structure. A standard strategy for dissecting the energetics of DNA-protein interactions is to mutate the complementary surfaces and to determine the change in binding free energy, in enthalpy, or in heat capacity. The changes are often difficult to interpret in terms of interactions between individual groups on the protein and DNA, since these thermodynamic measurements contain little direct information about concomitant changes in complex structure. The simultaneous measurement of the change in the number of sequestered water molecules at the DNA-protein interface can help bridge this gap.

Statistical Mechanics of Molecular Assemblies. When does the behavior of large molecules or assemblies such as DNA or membranes or fibrous proteins, as determined in cm-sized test-tubes, apply to the confines of a virus or a cell? While there have been important indications of significant differences, not until recently has there been a strategy to examine this potentially important question. During the past year, we have developed an explanation of the vapor-pressure paradox where lipids take up less water from a vapor than from liquid with the same activity. This theory is now guiding a six-lab experimental undertaking to examine the effects of confining surfaces, such as viral or cell walls, on molecular motion and packing. At the same time, we have developed a new theory of ionic charge fluctuation (van der Waals) forces simultaneously acting among assemblies rather than between pairs of molecules.

DNA Liquid Crystals. We have investigated the DNA phase diagram using osmotic stress to set the chemical potential of the DNA. So far, we have found that at high DNA densities there is a bond-orientational ordered phase with a liquid-like positional order (line-hexatic). It has long been speculated that such a phase should exist for long semi-flexible polymers. Our results are especially instructive because this system is analogous to magnetic flux line arrays in type II superconductors. At lower osmotic pressures and therefore lower densities, the pressure-density curves are dominated by fluctuation-enhanced interactions. Strong positional fluctuations lead to entropic repulsion, which effectively quadruples (at lower densities) the Debye screening length.

Adrian Parsegian

Forces Between Alpha-Helices and Between Telomere Analogs. Many proteins consist partly or exclusively of closed-packed bundles of alpha-helices. The thermodynamic stability of these bundles is obviously determined by the interplay of forming an alpha-helix and the specific and non-specific interactions between them. By learning about the details of these interactions (electrostatic, hydration, and steric), we will gain insight into protein folding and the relevant thermodynamics. This research focuses on the molecular interaction between alpha-helices at small intermolecular distances.

A similar set of measurements has begun, in collaboration with a group in Italy, on spontaneously forming four-stranded helices of guanosine monophosphate. The stability of these strands as well as the interaction between them shows strongly specific response to the nature of the ions in the surrounding medium. As they approach contact, the helices repel by hydration forces that reflect the structure of the helix as well as the perturbation of solvent water around the molecules.

Influence of Polyunsaturation on Lipid Membrane Elasticity. Cell membranes may undergo changes in area (per molecule) and curvature while maintaining bilayer integrity and fulfilling membrane barrier function. Some membranes are subject to extreme deformation, e.g., the erythrocyte membrane in capillary blood flow, and membranes involved in endo- and exocytosis. Further, the lipid matrix may adjust to conformational changes of integral membrane proteins by changing lipid area (per molecule) and membrane thickness. Lateral compressibility of membranes is likely to be important for proper cell function, and nature may control it by appropriate membrane composition. The influence of fatty acid polyunsaturation on neural membrane properties has been investigated before, and there is evidence that membranes rich in transmembrane receptors have particularly high concentrations of polyunsaturated fatty acids. The elastic area compressibility modulus, K_a, of lamellar liquid crystalline bilayers was measured using a new experimental approach involving ^2H NMR

order parameters of lipid hydrocarbon chains together with lamellar repeat spacings determined by x-ray diffraction. The combination of NMR and x-ray techniques allows accurate determination of lateral area per lipid molecule. The saturated stearic acid chain appears to be far less compressible than the polyunsaturated docosahexanoic acid chain.

Novel X-Ray Optics for Protein Structure Determination: NASA/NIH X-Ray Lens. This was the first year of an interagency agreement with NASA to test and develop their new optical fiber x-ray lens. With the ability to expose the same samples to x-rays directly from local generators and from the Brookhaven synchrotron, we have been able to obtain complementary structural information and to compare the strength and convenience of different sources. Samples will include those prepared for intermolecular force measurement and for observation of molecular assemblies.

BIOPHYSICS OF MESOSCOPIC ION CHANNELS

The Unit on Molecular Transport, headed by ***Sergey Bezrukov***, focuses on biomolecular and biophysical mechanisms of transmembrane transport through ion channels. Protein channels with an aqueous pore of 1 to 3 nanometer diameter, 'mesoscopic' channels, are of special interest. These membrane-bound structures allow cells to communicate with each other, to sense ligands, and to maintain homeostasis.

Channel structural equilibrium is extremely sensitive to the immediate environment including, for example, surrounding lipids or local acidity. To understand molecular transport properties, it is therefore important to study ion channels under conditions that reproduce their natural environment and, at the same time, to be able to manipulate these conditions under precisely controlled conditions. In this Unit, ion channels of diverse origins and biological functions are reconstituted into planar lipid bilayers to probe their characteristics in their various functional states.

By analyzing the statistical properties of channel-mediated ion currents, we address structure/function questions by using physicochemical approaches that are being developed for mesoscopic objects. Several of these approaches and techniques are unique to the Unit. The use of water- soluble molecular polymeric "probes" allows determination of the aqueous pore geometry and diffusion properties in various conformational states. Graded osmotic response, access resistance, and "molecular Coulter counter" approaches were all pioneered by the Unit. Frequency analysis of the electrical noise produced by reversible ionization of amino-acid residues in the channel links molecular structure with mechanisms of transport regulation. The Unit has also initiated studies on information transfer at the single-channel level with a special emphasis on stochastic resonance and the role of ambient noise in the biology and medicine of sensory transduction.

Probing Ion Channels with Soft Water-Soluble Polymers. The dynamics and free energy of polymers partitioning into a nanoscale pore were investigated using channels formed by *Staphylococcus aureus* alpha-toxin. Neutral linear polymers of polyethyleneglycol (PEG) were used in this work because of their well-studied physical and chemical properties, remarkably small persistence length, and commercial availability in a convenient range of molecular weights. Kinetic and thermodynamic parameters of polymer partitioning were determined by measuring polymer-induced changes in the pore's ionic conductance.

The free energy of polymer confinement in the pore deduced from our analysis differs significantly from predictions of scaling theory. The scaling approach yields a much weaker dependence of partitioning on the polymer weight but, surprisingly, gives a correct estimate for the polymer size corresponding to one kT of energy. The characteristic size determined in this manner distinguishes between polymers that partition into and those that are entropically excluded from the pore. Experiment shows that the free energy of polymer confinement is proportional to the third power of its weight, instead of scaling theory's first power. Moreover, the polymer-induced conductance fluctuations show a striking nonmonotonic dependence on the polymer molecular weight. The movement of polymer inside the pore is characterized by a diffusion coefficient that is orders of magnitude smaller than for polymer in the bulk aqueous solution.

Our results also suggest that PEG has an attractive interaction with the pore. This is in striking contrast to what one would expect if the pore were lined exclusively with hydrophilic amino acids. Recent work by others shows that PEGs are excluded from proteins in their native conformation yet can associate with proteins in their denatured states. Moreover, PEG appears to be excluded from hydrophilic regions of proteins and to be attracted to their hydrophobic segments. Thus, the water-filled alpha-toxin pore may, to some extent, be lined with

hydrophobic residues. This challenges the conventional belief that an aqueous pore of an ion channel can contain only hydrophilic residues. If our conclusion is correct, then the function of these residues is intriguing. One may speculate that they provide a mechanism to disrupt the water structure inside this pore to facilitate the transport of solutes.

Generally, questions of the structure-function relationship for ionic pores are addressed in terms of the pore's ability to conduct different ions. Recently, evidence for a pore's role in the transmembrane transport of biopolymers, such as proteins, was obtained. The present work demonstrates that a simple nonelectrolyte polymer can be used to probe a protein pore not only in terms of its physical size but also in terms of its functional properties. It is likely that different types of polymers will elicit characteristic channel-polymer interactions that are related, but not restricted, to the configuration of the polymer.

Reversible Ionization of Amino Acid Residues as a Structural Tool in Ion Channel Studies. It was previously shown that reversible ionization of amino acid residues by protons inside the channel's aqueous pore is a source of measurable noise. Frequency analysis of this noise yields the number of ionizable residues, their pKs, their kinetic parameters, and their tentative positions in the pore. Using this technique, the question of regulation of whole-cell currents by acidity of the media is addressed. In previous work by others, the apparent pK of 6-7 and steep conductance dependence on pH were attributed to the titration of histidine residues of membrane channels coupled with some form of cooperativity between channel-forming subunits. In this study, a pH dependence with a Hill coefficient of 3 was found for a single channel composed of one protein molecule. The experiments were carried out with mitochondrial ion channels, voltage-dependent anionic channels (VDAC), of various origins, reconstituted into planar lipid membranes.

Sergey Bezrukov

The electrophysiological properties, including single-channel conductance, anion selectivity, and voltage dependence have been shown to be identical for VDAC isolated from *Neurospora crassa* and the yeast *Saccharomyces cerevisiae*, suggesting that these channels have similar three-dimensional structures, in spite of their low primary sequence homology. The presence of an extra histidine within a tentative pore structure in *N. crassa* VDAC, vs. VDAC from yeast, makes it promising to compare pH-dependent properties of the two channels.

It was found that the low frequency open channel noise from VDAC in *N. crassa* exhibits a clear peak between pH 5.5 and 7.0. This correlates well with a change in single-channel conductance and with an unusually sharp ion dependence of selectivity on buffer acidity around pH 6. The relation between selectivity and pH is well described by a pK of 5.8 and a Hill coefficient of 3. In contrast, in the case of VDAC from yeast, the noise peak is absent, there is no detectable change in single-channel conductance, and the dependence of selectivity on pH is much weaker.

A first-order reaction model of reversible protonation at one ionizable site yields an effective pK = 5.8 and rate constants that are of the same order of magnitude as the rate constants of proton self-dissociation in water. However, the sharpness of the noise peak or of the selectivity change suggests a significant cooperativity already at the level of a single monomeric ion channel. We speculate that the mechanism of this cooperativity is related to protein flexibility to allow structural changes that are highly sensitive to the charge state of ionizable residues and, therefore, to pH.

Information Transduction in Voltage-Dependent Ion Channels in the Presence of Noise. Noise-facilitated signal transduction or the "stochastic resonance" (SR) phenomenon, originally proposed as an explanation for the periodic recurrences of the Earth's ice ages, has been attracting rapidly growing interest from researchers working in different areas of science, from physics to biology. Only a few years ago, it was generally accepted that SR can occur only in dynamic systems subjected to random forcing. Later, it was hypothesized, and then shown both experimentally and theoretically, that the simplest "stochastic resonator" consists only of signal, noise, and a threshold device.

Present work introduces yet another class of systems in which a noise-induced increase in the output signal-to-noise ratio (SNR) can be observed. These systems are both non-dynamic and threshold-free. SR is found in a very general model: a random pulse train in which the probability of pulse generation is exponentially dependent on an input that is composed of a sine-wave signal plus random noise. Analysis of the statistical properties of such a pulse train yields the following features of signal transduction: (1) threshold-free response, i.e., the ability to transfer small signals with a transduction coefficient independent of signal amplitude; (2) noise- facilitated signal transduction, i.e., the property to increase the output signal amplitude by adding noise to the system input; (3) noise- induced improvement in the output SNR, i.e., the existence of particular input noise levels that optimize the output signal quality.

The model offers a quantitative description for the experimental results found for voltage-dependent ion channels reconstituted into an artificial planar lipid bilayer membrane. It was found that introduction of bandwidth-restricted "white" noise to the holding potential facilitates signal transduction, i.e., ion channels show stochastic resonance. Specifically, addition of 10 - 20 mV (r.m.s.) noise to a small sine-wave input signal increases the output signal by about 20 - 40 dB, conserving and even slightly increasing the signal quality at the system output. The present theory (which is formulated for small and low-frequency signals) adequately describes our main experimental findings. In particular, it gives a good estimate for the input noise intensity corresponding to the maximal output SNR as well as for the SNR value itself.

Moreover, the theory predicts the existence of SR in all systems that can be represented by a random pulse train with exponential statistics. It shows that the two major SR attributes, noise-induced increase in signal transduction and improvement of the output SNR at some optimal noise value, are inherent properties of these systems. Since random pulse trains describe almost all physicochemical processes at the molecular level, stochastic resonance is probably a much more universal phenomenon than was previously believed.

PHYSICAL PRINCIPLES OF BIOMOLECULAR RECOGNITION, SELF-ASSEMBLY, AND REGULATION

The Unit on Molecular Forces and Assembly, headed by ***Sergey Leikin***, seeks to understand the mechanisms of biomolecular recognition and assembly and to determine the forces that organize proteins and regulate their stability and function. Recent force measurements revealed that the traditional concept of biomolecular recognition, assembly, and regulation via electrostatic interactions, hydrogen bonding, and hydrophobic contacts might be too simplistic. To improve on the current situation, this Unit works to develop a theory and measure interactions between helical macromolecules, whose interactions are vital to many recognition and assembly reactions in living organisms. Helix-helix interactions were measured in columnar phases of DNA, collagen, guanosine helices, and several polysaccharides. There is special emphasis at present on collagen assembly, with the intention to establish physical principles of collagen fibrillogenesis and collagen fiber stability. Collagen is the most abundant helical protein in the human body. Abnormal collagen-collagen interactions are responsible for severe pathology of human development (*osteogenesis imperfecta*) and for severe disease complications (e.g., connective tissue failure in diabetes). Despite a wealth of existing data on collagen fibrillogenesis and fiber stability, the nature of forces between collagen helices remains unclear. Understanding the physics of these interactions is clearly important for numerous biomedical applications and it is likely to lead to useful insights for solving more general problems of protein folding and assembly as well.

Theory of Electrostatic and Hydration Forces Between Helical Molecules. DNA, α-helical domains of proteins, collagen, actin filaments, and even some viral particles (e.g., tobacco mosaic virus) are all examples of biological helices. Helix-helix forces control protein folding and assembly, DNA packing, protein-DNA interactions, connective tissue formation and stability, and many other recognition and assembly reactions. Direct measurement of these forces allows us to probe the most basic molecular processes responsible for normal function and pathology in living organisms. The forces observed to date are very different from those predicted by traditional theories and which are built into all computer programs currently used for biomolecular modeling and drug design. By combining the measurements with rigorous physical theories we hope to create a solid foundation for better understanding of these fundamental processes and for employing this understanding in biomedical applications.

During the past year, we have derived exact, analytical expressions for the interaction energies valid for any patterns of discrete, charged surface residues. We demonstrated, e.g., that spontaneous aggregation of helices may occur even without full neutralization of the charge by bound counterions, as is sometimes observed in

counter-ion induced DNA condensation. Even under the most favorable aggregation conditions, an exponentially decaying image force prevents complete dehydration of the molecules, as is the case in aggregates of DNA, four-stranded guanosine helices, or collagen. The characteristic length of this short-range repulsion is determined by the helical pitch and by the number of helical strands. The values of this length predicted for DNA, guanosine helices, and collagen are in excellent agreement with the measured ones.

Symmetry Laws for Helix-Helix Interactions. The structures of many helical molecules, including DNA, were solved by applying symmetry constraints to x-ray diffraction patterns. It was not recognized, however, that similar constraints determine essential features of helix-helix interactions. Our theory showed that such forces are sums of interaction modes associated with different spatial periodicities of the helical charge pattern (just as x-rays diffraction produces a collection of spots corresponding to different spatial periodicities of the helical electron density pattern). We derived selection rules for these interaction modes from invariance of different charge patterns with respect to different symmetry transformations. One of the rules, e.g., dictates that two helices should interact more favorably when they have an integral rather than a non-integral number of charged residues per helical turn. Detailed calculations demonstrated that a change in the electrostatic interaction energy associated with this symmetry effect is sufficient to overcome the torsional rigidity and to induce the observed *B*-DNA "overwinding" from ~10.5 bp/turn in dilute solution to 10 bp/turn in fibers.

Hydration Forces Between Collagen Triple-Helices. It was long believed that the assembly of collagen triple helices into fibers is controlled by "salt bridges" and by interactions between non-polar amino acid side chains. Our direct force measurements, however, did not confirm this hypothesis. The data are inconsistent with both the electrostatic and hydrophobic interpretations. Instead, applying the theory of helix-helix forces, we discovered that the measured interactions are likely to be associated with the protein backbone hydration. Further experiments demonstrated that the character of collagen fiber swelling in non-aqueous solvents correlates with the solvent's ability to form a three-dimensional hydrogen-bond (HB) network, but not with the solvent's dielectric properties nor with its affinity for non-polar amino acids. Simultaneous force measurements and Raman spectroscopy revealed a rearrangement of the water HB-network upon decreasing separation between the helices in collagen fibers. The energetic cost of the rearrangement estimated from the Raman data agree well with the measured forces. The evidence obtained in all these experiments combined with the theory and with the evidence accumulated by other researchers leaves little doubt that the observed forces result mostly from the energetic cost of rearranging the HB-network in the water separating the collagen molecules.

Sergey Leikin

Sugar and Polyol Interfere with the Assembly and Reduce the Stability of Collagen Fibers by Disrupting Intermolecular Hydrogen-Bonded Water Clusters. By combining traditional measurements of thermodynamics and kinetics of collagen fiber assembly with direct force measurements, we revealed a remarkable specificity of collagen-collagen interactions to various sugars and polyols. This specificity is most strongly manifested in the drastic difference between the effects of 1,2-propanediols and 1,3-propanediols on the fibrillogenesis. While the former practically affects neither the forces nor the fiber assembly, the latter eliminates the intermolecular attraction and inhibits fiber formation. Detailed measurements suggested that, depending on the specific positions and conformation of their hydroxyls, some sugars and polyols may replace water in hydrogen-bonded clusters near some, still unknown recognition sites on collagen. Apparently, this disrupts water bridges between the opposing collagen helices, resulting in the loss of the intermolecular attraction and inhibition of the fibrillogenesis.

Collagen-Collagen Forces and Connective Tissue Pathology. We are working to apply the lessons from our studies of forces between collagen helices to improve our understanding of the factors controlling pathology of connective tissue development and contributing to various diseases. (1) Our study of the effect of sugars and polyols on these forces suggested that disruption of hydrogen-bonded water clusters due to slow accumulation of an open-chain glucose covalently attached at or near the recognition sites on collagen might be the main cause of fiber damage and connective tissue failure in diabetes. (2) The observed pH dependence of collagen-collagen forces indicated some involvement of histidines in the collagen-collagen recognition and fiber

assembly. Further measurements showed a striking direct effect of Zn^{2+} and Cu^{2+} on collagen fibrillogenesis, observed even at sub-physiological concentrations of these ions. Connective tissue pathology associated with zinc and copper ion deficiency (causing abnormal growth in children, slowing healing of wounds, helping proliferation of certain cancers, etc.) is well documented in the medical literature. It is believed to be associated with zinc- and copper-ion-dependent enzymes, but the possibility of a direct effect of the ions on connective tissue formation and stability has never been explored. (3) We are currently evaluating the possibility of direct measurement of forces between mutant collagens or their peptide models in order to gain a better insight into the causes of *osteogenesis imperfecta*.

LABORATORY OF PHYSICAL AND STRUCTURAL BIOLOGY

Chief
V. Adrian Parsegian, Ph.D.

Tenure-Track Investigators
Sergey Bezrukov, Ph.D.
Sergey Leikin, Ph.D.

Senior Fellow
Rudolf Podgornik, Ph.D.

Post-Doctoral Fellows
John Chik, Ph.D.
Per Lyngs Hansen, Ph.D.
Natalia Kouznetsova, Ph.D.
Tatiana Rostovtseva, Ph.D.
Nina Sidorova, Ph.D.
Helmut Strey, Ph.D.

Guest Researchers
Muriel S. Prouty, Ph.D.
George Walrafen, Ph.D.

ANNUAL BIBLIOGRAPHY

Bezrukov SM. The status of 1/f noise research in biological system: empirical picture and theories. In: Doering C, Kiss LB, Schelsinger M, eds. Proc. First International Conference on Unsolved Problems of Noise, 1997;243-54.

Bezrukov SM, Kasianowicz JJ. The charge state of an ion channel controls neutral polymer entry into its pore. European Biophys J 1997;26:471-6.

Bezrukov SM, Vodyanoy I. Signal transduction across alamethicin ion channels in the presence of noise. Biophys J 1997;73:2456-64.

Bezrukov SM, Vodyanoy I. Stochastic resonance at the single-cell level. Nature (London) 1997;388:632-33.

Bezrukov SM, Vodyanoy I. Stochastic resonance in non-dynamical systems without response thresholds. Nature (London) 1997;385:319-21.

Bezrukov SM, Vodyanoy I, Brutyan RA, Kasianowicz JJ. Dynamics and free energy of polymers partitioning into a nanoscale pore. Macromolecules 1996;29:8517-22.

Bezrukov SM, Vodyanoy I, Pustovoit MA. Noise-facilitated signal transduction in threshold-free non-dynamical systems: theory and experiment. In: Claeys C, Simoen E, eds. Noise in physical systems and 1/F fluctuations, World Scientific 1997;369-72.

Kas J, Strey H, Tang JX, Finger D, Ezzell R, Sackmann E, Janmey PA. F-actin, a model polymer for semiflexible chains in dilute, semidilute and liquid crystalline solutions. Biophys J 1996;70:609-25.

Kornyshev AA, Leikin S. Theory of interaction between helical molecules. J Chem Phys 1997;107:3656-74.

Kornyshev AA, Leikin S, Sutmann G. Overscreening in a polar liquid as a result of coupling between polarization and density fluctuations. Electrochim Acta 1997;42:849-65.

Koenig B, Strey HH, Gawrisch K. Membrane lateral compressibility measured by NMR and x-ray diffraction-effect of acyl chain polyunsaturation. Biophys J 1997;73:1954-66.

Kuznetsova N, Rau DC, Parsegian VA and Leikin S. Solvent hydrogen-bond network in protein self-assembly: solvation of collagen triple helices in nonaqueous solvents. Biophys J 1997;72:353-62.

Lasic DD, Strey HH, Podgornik R, Stuart MCA, Frederik PM. DNA cationic liposome complexes: structure and structure-activity relationships. J Am Chem Soc 1997;119:832-3.

Leikin S, Kozlov MM, Fuller NL, Rand PR. Measured effects of diacylglycerol on structural and elastic properties of phospholipid membranes. Biophys J 1996;71:2623-32.

Leikin S, Parsegian VA, Yang WH, Walrafen GE. Raman spectral evidence for hydration forces between collagen triple helices. Proc Natl Acad Sci USA 1997;94:2689-97.

Parsegian VA. Harness the hubris: useful things physicists might do in biology. Physics Today 1997;23-7.

Podgornik R. Isotropic: nematic transition of surface embedded polymers and the associated tubulization transition of the embedding surface. Phys Rev E 1996;54:5268-77.

Podgornik R. Orientational ordering of polymers on fluctuating flexible surfaces. Phys Rev Lett 1996;52:5170-7.

Podgornik R, Parsegian VA. On a possible microscopic mechanism underlying the vapor pressure paradox. Biophys J 1997;72:942-52

Podgornik R, Strey HH, Rau DC, Parsegian VA. Bond orientational order, molecular motion and free energy of high intensity DNA mesophases. Proc Natl Acad Sci USA 1996;93:4261-66.

Sidorova NY, Rau DC. Differences in water release for the binding of EcoRI to specific and nonspecific DNA sequences. Proc Natl Acad Sci USA 1996; 93:1.

Strey HH, Parsegian VA, Podgornik R. Equation of state for DNA liquid crystals: fluctuation enhanced electrostatic double layer repulsion. Phys Rev Lett 1997;78:895.

Templeton NA, Lasic DD, Frederik PM, Roberts DD, Strey HH, Pavlakis GN. Novel DNA: liposome complexes for increased systemic delivery and gene expression. Nat Biotechnol 1997;15:647-52

LABORATORIES OF THE SCIENTIFIC DIRECTOR

DNA MUTAGENESIS, REPAIR, AND REPLICATION

It is evident that genetic disorders, birth defects, and cancer are all consequent to aberrant DNA replication, mutations, or other lesions that compromise the molecular anatomy of the genome. However, genomic fidelity also has implications for evolution; without mutations there is no evolution, but too rapid a mutation rate means that a species will quickly cease to exist. Normally, DNA replication is a highly accurate process; in *Escherichia coli*, only one error occurs in every 10^{10} bases replicated. Much remains to be learned about the mechanisms that maintain this remarkable degree of fidelity, and this Section, led by ***Arthur Levine***, is focused on the molecular mechanisms of DNA replication, repair, and mutagenesis both in eukaryotic and prokaryotic cells.

Mutagenesis in Bacteria: the *umuDC* Operon. During the past year, ***Roger Woodgate***, who heads the Unit on DNA Mutagenesis, has continued to work on a prokaryotic mutagenesis system, of particular interest since the genes that promote mutagenesis in bacteria have been much more clearly defined than those in eukaryotes. When *E. coli* is treated with DNA damaging agents, e.g., ultraviolet radiation (UV), RecA is "activated" (a conformational change in which globular RecA becomes filamentous); activated RecA (RecA*) then mediates the proteolytic cleavage of LexA protein, the repressor of the multi-gene "SOS" pathway. This results in the induction of a number of genes whose products are directly or indirectly involved in DNA repair and mutagenesis. Generally, the organism first attempts to repair DNA lesions in an error-free manner. However, if this pathway fails, a second component of the SOS system, the tightly repressed *umuDC* operon, is induced, and the products of the *umuDC* genes allow the DNA replication machinery to transit unrepaired sites of damage. This "trans-lesion" synthesis is, however, error-prone; mutagenesis may be the cost of continued survival (of course, some mutations could promote survival). DNA polymerase III holoenzyme, RecA*, and the UmuDC proteins are all directly required for mutagenic trans-lesion synthesis, but the precise mechanism by which the UmuDC and RecA* proteins promote mutagenesis is unknown. In previous work, Woodgate and his coworkers found that the *E. coli* UmuD protein must be proteolytically processed to its active, truncated form, UmuD′, and that this cleavage is mediated by RecA*. This group has also found a physical association between RecA* and UmuD′, suggesting a mechanism by which the Umu proteins could be specifically targeted by RecA* to DNA lesions (RecA* filaments wrap around damaged DNA). Thus, this protein-protein-DNA targeting interaction is a third role of RecA in mutagenesis (cleavage of the LexA repressor of umuDC and UmuD cleavage being the other two roles).

To increase our understanding of the mutagenic process, we sought to determine the molecular structure of the mutagenically active *E. coli* UmuD′ protein. Recently, in a collaboration with Wayne Hendrickson (Columbia), this protein was crystallized; the structure was defined to a resolution of 2.5Å, and analyses revealed that the protein adopts an apparently novel fold, in that it is a tight β-structure with an extended amino terminus and a single α-helix encompassing residues 39 to 45. The seven β-strands are connected by short loops, and are oriented to form three β-sheets, which form the main body of the protein. The crystal is formed by two different kinds of protein interactions. In addition to UmuD′ forming a molecular dimer with itself, the amino terminal of UmuD′ can interact with the amino terminal of another "molecular dimer" to form an extended polymer ("filament") structure. While deletion of the N-terminal of UmuD′ still allows the protein to form the "molecular dimer," it precludes the formation of the polymer and greatly reduces the ability of UmuD′ to bind to the RecA nuclear protein filament, explaining why cells expressing this mutant are nonmutable. During the past year, we solved a second crystal structure of UmuD′, which is similar to the first but with more extensive carboxyl-terminal ordering. In recent experiments with purified wild-type as well as N-terminally deleted UmuD′, we concluded that the molecular dimer is indeed the dimer found in solution. Moreover, further evidence that the UmuD′ filament found in the crystal structure has biological significance arose from the observation that mutations at position 138 result in a gain of function (i.e., higher levels of UV-induced mutagenesis); this residue is in the region of the extended filament, and it seems likely that these mutant variants make better contacts between UmuD′ and the RecA or UmuC proteins, thus yielding a more active complex.

In other work during the past year, we identified *umu*-complementing genes on two incL/M plasmids, R471a and R446b. Molecular analysis of these genes revealed that they have a closer structural and functional relationship to *mucAB* (a plasmid form of *umu*) than to other members of the Umu-like family. (To date, the nucleotide sequence of 10 *umu*-like operons has been reported; these operons reside in both chromosomal and

episomal locations.) The two *umu*-like operons from R471a and R446b are very similar, but they have unusual insertion element polymorphisms within the promoter and terminator regions. We identified an insert, 5' to $mucAB_{(R471a)}$, that appears to be a novel retroelement encoding a putative reverse transcriptase (RT); this RT is related to the reverse transcriptases encoded by group II introns but it is embedded in a retron-like context. This observation suggests that the *mucAB*-like gene in R471a is located within a region of the R-plasmid that perhaps was once (or still is) a mobile genetic element and may explain the distribution of *umu*-like genes on R-plasmids and bacterial chromosomes, and how the *umu*-like genes might have moved from one location to another.

***RAD 30*: a Umu Homolog in Yeast.** Since many DNA repair processes are structurally and/or functionally conserved between prokaryotes and eukaryotes, and in particular, because this group and others have found evidence of such conservation in the Umu-like family, Umu homologs were sought during the past year in the yeast *Saccharomyces cerevisiae*. The entire nucleotide sequence of this yeast was translated and screened, with the result that an open reading frame was located on chromosome IV, which shares significant homology with the prokaryotic UmuC-like proteins. We found that disruption of this open reading frame results in modest UV sensitivity, and we therefore designated this new gene *RAD30* (*RAD30* is also related to *E. coli dinB* and the *S. cerevisiae Rev1* genes). The gene was cloned, and UV sensitivity as well as UV-induced reversion frequency were examined in mutant strains. While the *RAD30* mutant strains are indeed more sensitive to UV light than wild-type strains, there is no obvious defect in UV-induced reversion. Various double mutant strains were constructed and examined for UV survival, with the finding that *RAD30* normally participates in a novel post-replication, error-free repair pathway, which is dependent on *RAD6* and *RAD18*, but independent of *REV1, REV3, REV7* and *RAD5*. Other experiments revealed that *RAD30* is induced about 3.5 fold after treatment with UV light, and in this respect, it is similar to the *E. coli umuC* and *dinB* genes, both of which are damage-inducible.


Arthur Levine (left) and Roger Woodgate

A LexA Homolog in *Bacillus subtilis*. In other studies focusing on the conservation of repair/mutagenesis pathways, we purified and characterized a homolog of the *E. coli* LexA protein found in *Bacillus subtilis*, DinR. We found that purified DinR undergoes an autodigestion that is similar to that of LexA and that this cleavage reaction is promoted by *E. coli* RecA protein, again as with LexA. Moreover, purified DinR binds to the proposed *B. subtilis* SOS consensus sequence, consistent with the hypothesis that the *B. subtilis recA* gene is under negative transcriptional regulation. DinR recognizes and binds to specific sequences within the operator/promoter region of this *B. subtilis* SOS gene. Taken together, these data lead to the conclusion that the role of DinR is to act as the transcriptional repressor of the *B. subtilis* SOS regulon.

Our future work will focus on regions of the UmuD' protein that are important for interactions with both RecA* and UmuC, with the goal of providing a better understanding of how all of these proteins interact to form the so-called "mutasome." A second major thrust will be to pursue the molecular mechanisms that regulate and limit their post-translational expression. Finally, we will continue to explore the possibility that related proteins are involved in similar mutagenic processes in other enteric bacteria and in even more evolutionarily evolved organisms, including humans.

Mammalian DNA Repair: a DNA Damage Specific-DNA Binding (DDB) Protein Complex. DNA repair is a multi-step process that removes structural lesions from genomic DNA. If not repaired, DNA lesions can lead to mutations and/or alteration of gene expression, and ultimately, to cell transformation or death. The significance of the DNA repair process for human health is well reflected in the rapidly increasing number of human visceral cancers now known to arise as a consequence of mutations in repair genes, as well as the heritable human skin disease, *xeroderma pigmentosum* (XP). The high susceptibility of XP patients to sun-induced skin cancer is strongly correlated with the hypersensitivity of their cells to UV light, and the decreased capacity of these cells to repair UV-induced photoproducts in DNA. Efforts to clone the genes that are defective in the various XP complementation groups and to identify their products, which might provide the key to a fuller understanding of normal repair processes in human cells, have only recently met with success.

A group led by ***Arthur Levine*** has used model DNAs containing UV-induced lesions to address the molecular mechanism of lesion recognition. We previously identified a constitutive DNA damage-specific, DNA binding (DDB) protein complex in human cells that is induced to high levels of binding activity by UV treatment. The DDB complex recognizes a specific UV-photoproduct (6-4 pyrimidine dimers); it is the first such damage-specific, damage-inducible DNA binding complex to be identified in mammalian cells. The M_r of the largest DDB subunit is 127 kDa; a 48 kDa protein is present as well. The function of the DDB complex *in vivo* is still uncertain. However, it is likely that the complex is utilized in some aspect of processing of UV-damaged DNA, probably the initial recognition of damage, since its activity is regulated by UV light but it has no catalytic activity. DDB activity is absent in the cells of XP (Group E) patients. Moreover, the activity is highly conserved among vertebrates (e.g., it is found in chicken, marsupials, rodents, and primates), suggesting an important function in higher eukaryotes.

Previously, we purified the p127 component of DDB and cloned its gene. A p127 homolog was also found in *Drosophila*, which may allow us to exploit the power of fly genetics to elucidate the function of p127. During the past year, we found that immediately following UV irradiation, the p127 component of DDB, as well as XPA, RP-A, and PCNA, are translocated in the nucleus from low to high salt chromatin, indicating that these proteins move to a tight association with damaged DNA. However, unlike XPA (which functions exclusively in the early recognition of various DNA lesions), only a small fraction of p127 protein is translocated to the high-salt chromatin. This result suggests that DDB may have another cellular function(s) in addition to its role in nucleotide excision repair (NER). Moreover, there appears to be a specific interaction between p127 and RP-A, both *in vitro* and *in vivo*. The DDB/RP-A interaction results in enhanced binding of both proteins to DNA, and the similarity of DDB/RP-A to XPA/RP-A interactions additionally supports the notion that DDB functions in the damage-recognition step of NER.

In other experiments, we found that, immediately after UV irradiation, DDB activity cannot be recovered from normal cells, suggesting that a DDB protein that moves to tightly bound chromatin may not be extractable from the cells until DNA repair is complete (DDB activity cannot be recovered from normal cells until 24 hours following UV irradiation). The kinetics of recovery of extractable DDB binding activity from XPA cells are very different: here, not only is recovery not possible immediately after UV irradiation, but at 48 hours, recovery is still not possible—consistent with the fact that NER fails to occur in these cells. Interestingly, recovery of extractable activity was rapid from Cockayne syndrome-A cells, consistent with the fact that, in this syndrome, the defect in NER is secondary to a failure only of transcription-coupled repair. Since only a small fraction of the genome is affected, little if any DDB remains bound to chromatin following irradiation. In XPC cells, following UV, there is again an immediate loss of recoverable DDB activity, and this activity is recovered slowly. This result is consistent with the fact that the XPC protein is not required for NER of actively transcribed DNA, although it is required for repair of the bulk genome. Taken together, these data suggest that whatever the function of DDB, it is required for repair of the whole genome and is not functioning only with transcribed DNA.

The Role of RP-A in DNA Repair. We attempted to confirm a recently published observation that RP-A specifically promotes NER in XPE cells. In a collaboration with Rick Wood (ICRF), using three newly identified XPE lymphoblastoid cell lines, we found that all three lines contain abundant RPA as well as p127/p48. Moreover, extracts from these cell lines are able to carry out excision of a platinated adduct in an *in vitro* assay using a naked DNA substrate, and the addition of purified DDB to the extracts does not alter the results. In contrast, the addition of purified RPA enhances NER in this assay not only in the XPE extracts, but in normal cell extracts as well, i.e., the effect of RPA, in contrast to the previous report, is non-specific.

p48 is the Regulatory Subunit of UV-DDB. Recently, another lab has reported that all three of the XPE patients previously reported in the literature have mutations in the p48 but not in the p127 nor in RPA genes, suggesting that it is p48 that underlies the XPE defect. We therefore examined p48 expression in their three new XPE cell lines, finding that while p48 is abundant and entirely intranuclear in normal cells, much less p48 is present in the XPE lines and that it is distributed between the nucleus and the cytosol. Normally, most p48 moves to the tightly bound chromatin fraction after UV irradiation. However, the amount of total cellular p48 is dramatically reduced following UV, whereas the total amount of p127 does not change. DDB activity is not recoverable from any of the nuclear fractions other than tightly bound chromatin following irradiation even though, by Western blotting, abundant p127 is present in these other fractions. These data suggest that p48 moves initially to damaged chromatin following UV, but soon thereafter, it is degraded and therefore might not be available to promote the binding of p127 to DNA—a previously demonstrated requirement. Finally, while p48 is degraded

immediately after UV irradiation, it is induced to supra-normal levels by 48 hours, exactly at the time that DDB binding activity is once again recoverable from an irradiated cell. These results strongly suggest that p48 is the regulatory subunit of DDB.

To follow the movement of p127/p48 in living cells following UV irradiation, a GFP (green flourescent protein)/wild-type p48 gene fusion product was transfected into COS cells, and it was found, as predicted, that the expressed protein is almost entirely localized within the nucleus in irradiated cells, with only a small amount in the cytoplasm. In contrast, a mutant p48 (with a 20-amino-acid substitution in the N-terminal region) fails to localize in the nucleus but instead demonstrates a strong cytoplasmic signal, which appears to be lysosomal in origin. These results are consistent with the proposal that p48 normally undergoes lysosomal degradation soon after the initiation of repair; the mutant may be especially susceptible to such degradation.

Surprisingly, this group found earlier that in an *in vitro* assay for NER reconstituted with all of the purified NER components, DDB is entirely unnecessary for repair when the substrate is naked DNA. However, microinjection of purified DDB protein into XPE cells fully corrects their repair defect. This paradox has led us to hypothesize that DDB is required for the repair of chromatinized, but not naked, DNA. Thus, our current objective is to study DNA repair in the context of chromatin—a largely unexplored area of DNA research but one that can potentially profit from lessons learned in the study of transcription of chromatinized vs. naked DNA.

SECTION ON DNA REPLICATION, REPAIR AND MUTAGENESIS

Chief
Arthur S. Levine, M.D.

Senior Investigator
Roger Woodgate, Ph.D.

Post-Doctoral Fellows
Ekaterina Chumakov, Ph.D.
Martín Gonzalez, Ph.D.
John McDonald, Ph.D.
Vesna Rapić Otrin, Ph.D.
Agnés Tissier, Ph.D.
Dominique Vandewiele, Ph.D.

Pre-Doctoral Fellows
Majid Cina
Erinn Maury
Kevin Winterling

ANNUAL BIBLIOGRAPHY

Avantaggiati ML, Ogryzko V, Gardner K, Giordano A, Levine AS, Kelly K. Recruitment of p300/cBP in p53-dependent signal pathways. Cell 1997;89:1175-84.

Carbone M, Rizzo P, Grimley PM, Procopio A, Mew DJY, Shridhar V, de Bartolomeis A, Esposito V, Giuliano MT, Steinberg SM, Levine AS, Giordano A, Pass HI. Simian virus-40 Large-T antigen binds p53 in human mesotheliomas. Nat Med 1997;3:908-12.

Efrati E, Tocco G, Bruck I, Eritja R, Woodgate R, Wilson SH, Tower J,Goodman MF. Biochemical analysis of possible deviations from the "A rule": specificity of nucleotide insertion at abasic DNA template lesions by pol β. In: Hagen U, Jung H, Streffer C eds. Radiation Research. New York:Taylor and Francis, 1996;2:324-31.

Lawrence CW, Gibbs P, Woodgate R, Borden A, Szekeres E, Nelson J,Hinkle D. Mutagenic mechanisms in yeast and *E. coli*: studies with individual UV photoproducts and abasic sites. In: Hagen U, Jung H, Streffer C eds. Radiation Research, New York:Taylor and Francis, 1996;2:320-3.

McDonald JP, Levine, AS, Woodgate R. The *Saccharomyces cerevisiae RAD30* gene, a homologue of *Escherichia coli dinB* and *umuC*, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. Genetics 1997;147:1557-68.

Rapić-Otrin V, McLenigan M, Takao M, Levine AS, Protić M. Translocation of a UV-damaged DNA binding protein into a tight association with chromatin after treatment of mammalian cells with UV light. J Cell Sci 1997;110:1159-68.

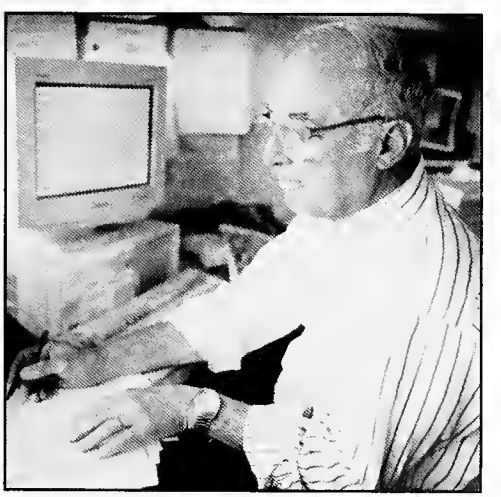
Watanabe-Akanuma M, Woodgate R, Ohta T. Enhanced generation of A:T-> T:A transversions in a *recA730 lexA51*(Def) mutant of *Escherichia coli*. Mutat Res 1997;373:61-6.

Winterling K, Levine AS, Yasbin RE, Woodgate R. Characterization of DinR, the *Bacillus subtilis* SOS repressor. J Bacteriol 1997;179:1698-703.

NERVE GROWTH FACTOR

The Section on Growth Factors, under the direction of ***Gordon Guroff***, has as its primary objective the study of nerve growth factor (NGF) at the biochemical, molecular, and cellular level and the mechanisms by which it and the other neurotrophins control the survival, development, and death of key neuronal populations. Nerve growth factor was the first recognized and is now the best characterized member of the neurotrophin family of peptide growth factors. The neurotrophin family, now five in number, supports a wide variety of neural cells. Nerve growth factor is required for the survival and development of sympathetic and sensory neurons. It also is involved in the support of several other cell types, including specific populations of neurons in the central nervous system, the cells of the adrenal medulla, and a number of tumors as well. The action of nerve growth factor on these different cells is initiated by its binding to specific receptors. There are two separate nerve growth factor receptors, one the product of the *trk* proto-oncogene and a second site known as p75. The binding of nerve growth factor to these receptors activates one or more signal transduction pathways that lead to alterations in the phosphorylation and, consequently, the function of key proteins in the cell and to changes in the expression of specific genes. These changes in protein function and in gene expression, caused by the changes in phosphorylation, are the mechanism by which nerve growth factor exerts its effects on its target cells. Much of the work leading to this concept has been done with the PC12 pheochromocytoma, a cell line derived from a tumor of the rat adrenal medulla. This clonal line continues to be one of the most informative tools available for the study of nerve growth factor and a key model for neuronal differentiation in general. In the presence of nerve growth factor, PC12 cells stop dividing, elaborate neurites, become excitable, and will synapse with appropriate muscle cells in culture. Indeed, they change from a rapidly dividing chromaffin cell to a terminally differentiated sympathetic neuron within a few days. The changes in phosphorylation that underlie these striking alterations in phenotype occur in virtually every compartment in the cell. Phosphorylation of nerve-growth-factor-stimulated calcium channels appears to regulate the calcium flux across the membrane and, in turn, the intracellular calcium levels. Nerve growth factor-induced phosphorylation of the elements that control protein synthesis, such as eIF-4E, eEF-2, and S6, almost certainly alters the rate and the specificity of the translation of various mRNAs. The nerve growth factor-induced phosphorylation of specific transcription factors, such as NGFI-B, determines which genes are expressed. An understanding of the action of nerve growth factor will surely illuminate the control of neuronal differentiation and survival. Clinical interest in these peptide factors is intense because of the role they might play in neurodegenerative diseases.

Nerve Growth Factor and Calcium Uptake. Nerve growth factor influences the uptake of calcium into PC12 cells. There is evidence that the strength of this action of nerve growth factor depends upon the levels of calcium in the cell at the time. Thus, nerve growth factor can be considered a calciostat, raising calcium levels when they are low, and lowering calcium levels when they are high. It has been suggested that, since the survival of neurons requires adequate intracellular calcium levels, this action may be one of the mechanisms by which nerve growth factor keeps neurons alive. Further, when neurons are exposed to adverse conditions, such as lack of glucose or oxygen as occur during a stroke, calcium levels rise leading to neuronal damage and death. Since nerve growth factor appears to prevent this increase, this action may be one of the mechanisms by which nerve growth factor prevents neuronal death. Recent studies with 3T3 cells transfected with nerve growth factor receptors have shown that both the high-affinity nerve growth factor receptor, $p140^{trk}$, and the low-affinity nerve growth factor receptor, $p75^{NGFR}$, will mediate nerve growth factor-stimulated calcium uptake into PC12 cells. Present work in the Section focuses on the mechanisms by which these two receptors act on calcium uptake. Ligands specific for each of these receptors are being used to evaluate these separate mechanisms. The related effect of K-252a, a kinase inhibitor acting on the $p140^{trk}$ receptor, is also being studied. The relevant signal transduction pathways are being identified through the use of appropriate mutants and inhibitors. Precipitating antibodies directed against the major calcium channels are being used to inspect the role of phosphorylation in this action of nerve growth



Gordon Guroff

factor. This work highlights the role of calcium in the mechanisms by which nerve growth factor promotes neuronal survival, neuronal protection, and neuronal plasticity.

Nerve Growth Factor and the Epidermal Growth Factor Receptor. The finding, made some years ago, that PC12 cells respond both to the differentiating agent nerve growth factor and to the mitogen epidermal growth factor, has led to important studies on comparative signal transduction and to equally significant experiments on receptor interplay. Considering the fact that differentiation with nerve growth factor causes the down-regulation of the epidermal growth factor receptor, and in an effort to understand the molecular basis of this down-regulation, we have focussed on a possible nerve growth factor-induced decrease in the transcription of the epidermal growth factor receptor gene. The levels of epidermal growth factor receptor mRNA have been measured by Northern blotting and by competitive RT-PCR, and we have detected nerve growth factor-induced decreases in mRNA levels with both methodologies. Since epidermal growth factor receptor mRNA stability is unchanged under these conditions, a decrease in transcription appears to be the cause of the decrease in mRNA levels. To confirm this, portions of the epidermal growth factor receptor promoter linked to a luciferase reporter have been transfected into control cells and into nerve growth factor-treated cells. Such experiments have revealed a profound decrease in transcription in cells treated with nerve growth factor. These decreases in transcription have been found to depend on the mediation of the $p140^{trk}$ receptor and the Ras/Raf/MAP kinase signal transduction pathway. A recently cloned inhibitory transcription factor, GCF-2, has been shown to increase during the decrease in epidermal growth factor receptor gene transcription, and this increase was also found to depend on $p140^{trk}$ and the Ras/Raf/MAP kinase pathway. In a related line of research, the signal transduction pathway controlling the nerve growth factor-induced down-regulation of the epidermal growth factor receptor itself has been identified. Using PC12 cells transfected with dominant-negative signaling elements, it has been shown that the Ras/Raf/MAP kinase pathway is responsible for this down-regulation as it is for neurite outgrowth itself. However, it is clear that epidermal growth factor receptor regulation is not linked to neurite outgrowth, because it occurs in nerve-growth-factor-treated cells that are prevented from attaching to a matrix and are, thus, not producing neurites. Further, it does not occur in cells treated, for example, with staurosporine, which do produce neurites but, as we have recently shown, by a different signal-transduction pathway than that serving nerve growth factor-induced neurite formation. These studies promise to elucidate the molecular mechanism by which the levels of epidermal growth factor receptor are controlled during nerve-growth-factor-induced differentiation. Parallel studies suggest that both erbB2 and erbB3, receptors that are homologous with the epidermal growth factor receptor and are of significant clinical interest because of their probable role in the initiation or promotion of tumor growth, undergo similar nerve growth factor-induced down-regulation. It is possible that their levels may be controlled by mechanisms similar to those regulating the levels of the epidermal growth factor receptor.

Nerve Growth Factor and the Transcription Factor NGFI-B. The transcription factor NGFI-B is induced by a number of different effectors in PC12 cells. Nerve growth factor is among these, but nerve growth factor also has the effect of inducing a robust phosphorylation of NGFI-B molecule. Previous work from this laboratory has shown that specific nerve-growth-factor-induced phosphorylation inhibits the binding of NGFI-B to its target sequence on DNA, presumably limiting its transcriptional activity. A kinase, termed NGFI-B kinase I, has been purified and characterized, and it has been shown to phosphorylate a specific serine in the DNA-binding domain of NGFI-B. The identity of this kinase is not known with certainty, but it is very similar to or may even be CREB kinase, also known as rsk2, the enzyme phosphorylating the protein that binds to the cAMP response element. In order to understand the biology of this apparently anomalous action of NGFI-B kinase, e.g., an enzyme induced by nerve growth factor that blunts the action of a transcription factor also induced by nerve growth factor, we have conducted experiments on the actions of other effectors that induce NGFI-B, but do not induce NGFI-B phosphorylation. It has now been shown that the induction of NGFI-B by depolarizing concentrations of K^+ causes increased transcription of a reporter gene linked to the NGFI-B response element. However, the addition of nerve growth factor as well inhibits this action of K^+. Furthermore, this action of nerve growth factor is clearly dependent on the phosphorylation, by NGFI-B kinase I, of a specific serine, Ser350, in NGFI-B. This phosphorylation inhibits the binding of NGFI-B to its response element and, as a consequence, inhibits the transcriptional activation that unphosphorylated NGFI-B displays. These data confirm the *in vivo* role of NGFI-B kinase, a role hitherto shown only in a cell-free system. They also highlight the importance of the nerve growth factor-dependent induction of NGFI-B kinase rather than of NGFI-B itself. Perhaps most importantly, they provide an example of the interaction of nerve growth factor with neuronal activity in the expression of specific genes. One interpretation of the data could be that at a certain developmental stage,

perhaps upon synapse formation, the role of neuronal activity in gene expression is altered by the availability of agents such as nerve growth factor.

SECTION ON GROWTH FACTORS

Chief
Gordon Guroff, Ph.D.

Post-Doctoral Fellows
Hao Jiang, Ph.D.
Yasuhiro Katagiri, Ph.D.
Elena Kazanova, Ph.D.
Michael Czar, Ph.D.
Edward Mills, Ph.D.
Xu-wen Liu, M.D., Ph.D.

Pre-Doctoral Fellows
Kuldip Hillyer
Alea Chabuk

Senior Technical Specialists
Geneva Dickens
Keiko Hashimoto

ANNUAL BIBLIOGRAPHY

Dickens G, Lavarreda M, Zheng WH, Guroff G. Involvement of protein kinase C in nerve growth factor- and K-252a-stimulated calcium uptake into PC12 cells. J Neurosci Res 1997;47:271-6.

Jiang H, Ulme DS, Dickens G, Chabuk A, Lavarreda M, Lazarovici P, Guroff G. Both p140(trk) and p75(NGFR) nerve growth factor receptors mediate nerve growth factor-stimulated calcium uptake. J Biol Chem 1997;272:6835-7.

Jiang H, Movsesyan V, Fink DW Jr, Fasler M, Whalin M, Katagiri Y, Monshipouri M, Dickens G, Lelkes PI, Guroff G, Lazarovici P. Expression of human p140trk receptors in p140trk-deficient, PC12/endothelial cells results in nerve growth factor-induced signal transduction and DNA synthesis. J Cell Biochem 1997;66:229-44.

Katagiri Y, Hirata Y, Milbrandt J, Guroff G. Differential regulation of the transcriptional activity of the orphan nuclear receptor NGFI-B by membrane depolarization and nerve growth factor. J Biol Chem 1997;272:31278-84.

Lazarovici P, Oshima M, Shavit D, Shibutani M, Jiang H, Monshipouri M, Fink D, Movsesyan V, Guroff G. Down-regulation of epidermal growth factor receptors by nerve growth factor in PC12 cells is p140(trk)-, Ras-, and Src-dependent. J Biol Chem 1997;272:11026-34.

PERINATOLOGY RESEARCH BRANCH

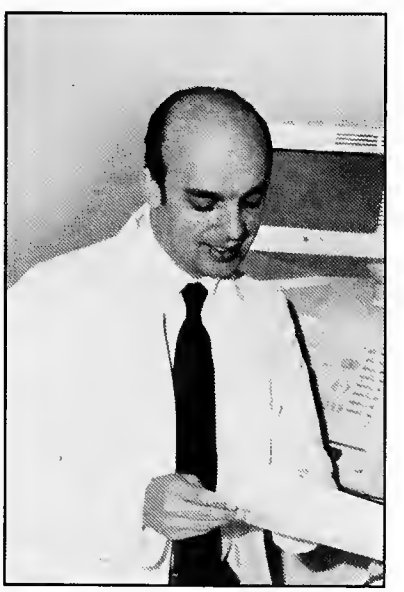
Roberto Romero, M.D., Chief

The Perinatology Research Branch conducts clinical and laboratory research in maternal and fetal diseases responsible for perinatal morbidity. The Branch emphasizes a multi-disciplinary approach, using expertise from clinical sciences (maternal fetal medicine, neonatology, and perinatal pathology), as well as basic sciences and epidemiology, to improve the etiologic understanding, diagnosis, treatment, and prevention of disorders related to infant mortality. Emphasis is placed on congenital anomalies and causes of prematurity. These two conditions are the leading causes of perinatal morbidity worldwide and are major contributors to infant mortality.

PATHOPHYSIOLOGY OF PREMATURE LABOR AND COMPLICATIONS OF PREMATURITY

The Branch continues its work in the pathophysiology of preterm labor. At least 75% of perinatal deaths not due to congenital abnormalities are associated with prematurity. Moreover, survivors are at an increased risk for long-term neurologic and developmental handicaps. Thus, prematurity is considered the single most important problem in obstetrics today. Despite the availability of tocolytic agents (drugs that stop uterine contractions) and programs to improve maternal nutrition and prenatal care, the preterm delivery rate has remained essentially unchanged at about 9%, representing 180,000 preterm births per year. Studies performed by our group indicate that at least 25% of all preterm births are associated with subclinical microbial invasion of the amniotic cavity. Women with subclinical intrauterine infections are refractory to tocolysis and at higher risk for perinatal complications and thus contribute significantly to perinatal morbidity and mortality associated with preterm birth. The studies conducted in this laboratory are designed to define the role of intrauterine infection in preterm labor, and to develop diagnostic and therapeutic modalities for the treatment of preterm labor.

The Fetal Inflammatory Response Syndrome. Microbial invasion of the amniotic cavity is present in 10% of patients with preterm labor and intact membranes and in 30% of patients with preterm premature rupture of membranes (PROM). Moreover, bacterial "footprints" have been detected in the amniotic fluid of as many as 80% of patients with preterm labor and intact membranes. Microorganisms in the amniotic cavity or maternal compartment may reach the human fetus and stimulate the biosynthesis of pro-inflammatory cytokines. Increased bioavailability of pro-inflammatory cytokines during fetal life may lead to the development of an acute phase response similar to that observed in adult patients with a "systemic inflammatory response syndrome", a serious condition characterized by multiple organ failure associated with sepsis. Therefore, outpouring of pro-inflammatory cytokines into the fetal circulation may make the fetus critically ill and also provide a signal for the onset of preterm parturition in the context of intrauterine infection. If this hypothesis is correct, fetuses with evidence of a systemic inflammatory response syndrome *in utero* should have a higher rate of perinatal morbidity than those without this condition, and deliver preterm. To address this question, a study was conducted of patients who had undergone amniocentesis and cordocentesis at Hutzel Hospital/Wayne State University in Detroit, Michigan. It was found that neonates who develop severe morbidity have higher concentrations of fetal plasma IL-6 than than healthy neonates (median 15.9 pg/ml, range 0.5-319.7 vs. median 5.4 pg/ml, range 0.9-69.1, respectively, $p < 0.005$). A plasma IL-6 cut-off value of 11 pg/ml was used to define the presence of a "systemic inflammatory response" in fetuses. The presence of "the fetal inflammatory response syndrome" was found to be associated with severe neonatal morbidity in 77.8% of cases, whereas such complications are observed in only 29.7% neonates without this syndrome ($p < 0.05$). Stepwise logistic regression analysis demonstrated that fetal plasma IL-6 concentration is an independent predictor of the occurrence of severe neonatal morbidity (odds ratio 4.3, 95% confidence



Roberto Romero

interval 1-18.5) after adjusting for gestational age at delivery, clinical chorioamnionitis, cordocentesis-to-delivery interval, amniotic fluid culture and amniotic fluid IL-6 results. Moreover, fetuses with a systemic inflammatory response syndrome have a shorter intrauterine stay after sampling than those without elevated fetal plasma IL-6. These findings suggest that the pathophysiologic derangements responsible for some neonatal complications traditionally attributed to immaturity begin before birth. Such an interpretation is consistent with other observations, previously reported by the Perinatology Research Branch, which indicate that an inflammatory process already present at birth may mediate short and long-term complications of prematurity, such as periventricular leukomalacia and cerebral palsy.

Microbial invasion of the amniotic cavity was found to be associated with the presence of a fetal inflammatory response syndrome [63.4% (26/41) vs. 14.9% (17/114), for fetuses with and without microbial invasion of the amniotic fluid, respectively, ($p < 0.01$)], suggesting that fetal exposure to microorganisms and their products may result in the elevation of plasma IL-6. However, a fetal inflammatory response syndrome was also detected in the absence of demonstrable microbial invasion of the amniotic cavity. Further studies are required to determine the etiology of this condition in the absence of demonstrable infection. Possibilities that need to be explored are infection that escaped detection by traditional microbiologic techniques (caused by viral, *Chlamydia*, and other fastidious microorganisms), or an inflammatory process other than infection.

The concept that the fetus of the mother presenting with preterm labor or preterm PROM may be critically ill, with a systemic inflammatory response syndrome, calls for a reappraisal of the current clinical management of these conditions. The standard approach of attempting to prolong intrauterine stay with pharmacologic inhibition of uterine contractility in the patients with preterm labor or expectant management of the patients with preterm PROM may be an unwise course of action if the fetus is ill. Rapid methods to identify the affected fetus are needed as well as interventions to prevent short-term and long-term handicaps.

Evidence of a Role of the Human Fetus in the Initiation of Preterm Parturition. A central role for the fetus in determining the duration of pregnancy by signaling the onset of labor has been postulated based upon the observations in sheep, in which teratogenic or experimental destruction of the fetal hypophysis/hypothalamus results in failure to initiate parturition. Similarly, human fetuses with severe disruption of the central nervous system (anencephaly) have an increased rate of prolonged gestation. However, a role of the human fetus in preterm parturition has not been established unequivocally.

In previous years, we have proposed that, in the setting of intrauterine infection, the host-fetus and/or mother signals the onset of labor by resorting to pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), interleukin-6 (IL-6) and interleukin-8 (IL-8). These cytokines are capable of inducing a complex set of biological phenomena, including a change in the phenotypic characteristics of myometrium, increased prostaglandin biosynthesis, degradation of extracellular matrix, and neuroendocrine phenomena that may lead to the activation of the common terminal pathway of parturition.

This year, the Perinatology Research Branch produced evidence that the human fetus plays a role in the initiation of preterm parturition. A cohort study was conducted on patients admitted with preterm premature rupture of membranes and who were not in labor on admission and who had undergone amniocentesis or cordocentesis. Fetuses with a systemic inflammatory response syndrome (fetal plasma IL-6 > 11 pg/ml) were seen to have a higher rate of spontaneous preterm delivery within 24, 48, and 72 hours of the procedure than those without this syndrome (56% vs. 24%; 81% vs. 36%; and 88% vs. 40%, respectively, $p < 0.05$ for each). Moreover, survival analysis indicated that fetuses with elevated plasma IL-6 have a shorter cordocentesis-to-delivery interval than those with normal plasma IL-6 [median: 0.8 days (range: 0.1-5) vs. median: 6 days (range 0.2-33.6), respectively, $p < 0.05$. Fetal plasma IL-6 is the only covariate significantly associated with the duration of pregnancy after adjusting for gestational age, amniotic fluid IL-6, and the microbiologic state of the amniotic cavity. These observations indicate that a systemic fetal cytokine response is associated with the impending onset of spontaneous preterm labor in patients with preterm PROM and therefore provide evidence for a role of the human fetus in determining the timing of the onset of preterm labor.

Evidence that the Injury Responsible for Bronchopulmonary Dysplasia May Begin *in Utero*. Bronchopulmonary dysplasia (BPD) is emerging as one of the most important sequela of prematurity. Among infants with birth weights below 1500 grams, 15-47% are diagnosed to have BPD and a fraction will have significant respiratory handicaps during childhood. Surfactant treatment and modern ventilatory support have had a major impact in the frequency and severity of respiratory distress syndrome. However, the frequency of BPD in very low birth weight infants has increased steadily in the last two decades from 10% to 32%. Affected infants are

at risk for long-term requirement of oxygen therapy and for persistence of pulmonary symptoms (i.e., wheezing, retractions, cyanosis, nasal discharge, etc.). Other serious long term complications include recurrent pneumonia, right and left ventricular hypertrophy and hypertension. BPD has also been identified as an independent risk factor for the subsequent development of cerebral palsy. Death has been reported to occur in 11-71% of infants with chronic lung disease.

BPD has been traditionally attributed to the injury of immature lung tissue inflicted by the combination of barotrauma and oxygen toxicity. However, this does not explain the development of BPD in infants with mild respiratory distress syndrome or the absence of chronic lung disease in some infants with severe hyaline membrane disease requiring prolonged and high pressure ventilation. Therefore, other unidentified factors may play a role in the genesis of chronic lung disease in the setting of prematurity. Recently, infection has been recognized as a potential etiologic factor for BPD. Nosocomial infections during the neonatal period and colonization of the neonatal respiratory tract with *Ureaplasma urealyticum* have been implicated in the pathogenesis of BPD through the participation of inflammatory cytokines. However, little consideration has been given to the possibility that the pathogenic process leading to chronic lung disease may begin during fetal life. The Perinatology Research Branch conducted a study to determine if exposure to inflammatory mediators *in utero* was a risk factor for BPD. Amniotic fluid interleukin-8 (IL-8) was measured in a group of patients with preterm labor and preterm premature rupture of membranes who had amniotic fluid retrieved by amniocentesis and delivered between 24-28 weeks of gestation. The median amniotic fluid IL-8 concentration was found to be higher in patients whose neonates subsequently developed BPD than in those who did not. This relationship remains significant after correcting for the effect of gestational age and birth weight (odds ratio: 11.9; $p < 0.01$). Color and spectral Doppler studies of fluid movement in the perinasal area demonstrated influx of amniotic fluid during fetal breathing. We propose that inspired amniotic fluid with a high concentration of pro-inflammatory cytokines and chemokines may reach the lower tracheo-bronchial tree of the fetus and elicit an antenatal inflammatory response. Indeed, neutrophils and macrophages have been demonstrated in the tracheo-bronchial aspirate obtained shortly after birth in infants who subsequently developed BPD. Further work is required to determine if early identification of infants at risk and treatment with biological response modifiers may alter the natural history of this disease.

Expression of Anti-Microbial Peptides in the Genital Tract. A central question in the prevention of preterm birth is why some women develop an ascending intrauterine infection and others do not. Under normal circumstances, the lower genital tract (vulva, vagina and cervix) is colonized with microorganisms but the amniotic cavity is sterile. Effective host defense mechanisms must prevent ascending microbial invasion from the lower genital tract into the uterus during pregnancy. The mucus plug (condensation of cervical secretions formed during pregnancy) has been thought to constitute a mechanical barrier against ascending infection. Moreover, cervical mucus can also inhibit microbial proliferation. The precise nature of antimicrobial factors responsible for this biological activity has not been elucidated. The discovery of broad-spectrum antimicrobial peptides within the bovine tracheal epithelium (tracheal antimicrobial peptide), murine small intestine (cryptdins), and Paneth cells of the human small intestine (defensin 5, defensin 6) prompted investigators in the Perinatology Research Branch to explore the possibility that defensins may be expressed in the genital tract. Messenger RNA from amnion, chorion, endometrium, endocervix, myometrium, placenta, small intestine, peripheral blood lymphocytes and from cervical, endometrial and trophoblast cell lines, was reverse-transcribed using a 3′ RACE adapter. 3′ RACE PCR was conducted using an upstream human defensin 5 primer and 3′ RACE adapter primer. PCR products hybridizing to a human defensin 5 probe were cloned for sequence analysis. Chorionic tissue, endocervical tissue, endometrial tissue, and an endometrial cell line all demonstrated a single hybridizing 362 base pair PCR product. Sequence analysis of all clones demonstrated near perfect identity with human defensin 5. Since some intra-uterine infections are acquired by transplacental passage and since this organ is rich in bone marrow-derived cells, we also explored whether human placenta expressed defensin 1, a hematopoietic defensin. Messenger RNA from term placenta was reverse transcribed using a 3′ RACE adapter. 3′ RACE PCR was conducted using conserved upstream human defensin primers (HD5UTs, HD44s) and a 3′ RACE adapter primer. Southern analysis of 3′ RACE PCR products was subsequently preformed using a conserved internal human defensin probe (SIG68a). Southern analysis demonstrated two hybridizing bands of approximately 468 bp and 300 bp in placenta but not a negative cDNA control. These findings suggest that natural antimicrobial peptides are expressed in the genital tract and placenta, a previously unrecognized mechanism of host defense against infection. Further studies are required to determine if abnormal expression of anti-microbial peptides in the lower genital tract, gestational tissues, and placenta may lead to intrauterine and transplacental infections.

Fetal Biometry in the Assessment of Fetal Age in the Mid Trimester of Pregnancy. Gestational age assessment is an integral part of prenatal care. The diagnosis of fetal growth disorders (e.g., fetal growth retardation and macrosomia) and the interpretation of many laboratory tests used in clinical obstetrics require accurate determination of the duration of pregnancy. The concentrations of maternal serum alpha feto-protein, estriol, and human chorionic gonadotropin, used for screening of neural tubes defects and chromosomal abnormalities during pregnancy, change as a function of age. Inaccurate age assignment results in an increase in the rate of false positive and false negative cases, diagnostic errors, unnecessary invasive procedures, and increased patient anxiety.

The standard method of assigning duration of pregnancy is menstrual age, which is based upon the knowledge of the first day of the last menstrual period (LMP). Accuracy depends upon the reliability of menstrual history. However, in this study as many as 45% of pregnant women had sub-optimal menstrual history because of uncertain recall, irregular menstrual cycles, bleeding in early pregnancy or oral contraceptive use within two months of conception. Consequently, alternative methods have been used to assign age. By far the most popular method is dating with ultrasound, which is based upon the premise that fetal size measured with ultrasound is an accurate indicator of gestational age.

Several biometric parameters of fetal size, including the biparietal diameter, head circumference, and femur length, have been used to predict gestational age. Most formulae describing the relationship between these fetal biometric parameters and gestational age have been generated in patients in whom the precise time of conception was not unknown and thus have an inherent imprecision. Formulae derived from pregnancies occurring as a result of *in vitro* fertilization (IVF) can greatly reduce this imprecision because the time of conception is known. The Perinatology Research Branch conducted a study in collaboration with the Cornell University Medical Center to determine the accuracy of fetal biometry between 14 and 22 weeks in the prediction of gestational age in singleton, twin, and triple gestations using a population of patients who conceived as a result of *in vitro* fertilization. This gestational age was chosen because most pregnant women in the United States undergo sonographic examination to screen for congenital anomalies during this window of time.

The results of this study demonstrated that the size of the fetal head circumference is the most accurate predictor of gestational age of all parameters studied. The addition of one parameter (abdominal circumference or femur length) or two (abdominal circumference and femur length) significantly improves the accuracy of the prediction based on head circumference alone. Although the improvement is marginal (less than 1 day), there are still reasons to use multiple parameters in clinical practice. First, the information is readily available because these measurements are obtained as part of routine ultrasound examination and computation is easily performed by microcomputers. Second, using more than one parameter allows quality control by identification of outliers due to biological phenomena (i.e., congenital anomalies or growth variation) or technical error in measurement of a single parameter.

Although the biparietal diameter (BPD) has been used as the standard method for dating with ultrasound for the last twenty five years, the findings of this study indicate that head circumference alone or in combination with one or two additional parameters is a better predictor of fetal age than the biparietal diameter. Inasmuch as the biparietal diameter and the head circumference (HC) are measured in the same plane and biparietal diameter (but not the head circumference) is affected by the shape of the head, there does not seem to be any justification to continue measuring the biparietal diameter. The ease of use of electronic calipers to measure the perimeters of the head has eliminated any objection to replacing BPD with HC as the standard dating parameter.

The clinical practice in most obstetrical units is that if discrepancy between gestational age by LMP disagrees by more than 14 days with that derived from fetal biometry in the second trimester, the LMP assessment is superseded by fetal biometry. Our study suggests that when a discrepancy of more than 7 days exists between the two, the biometric prediction should be given preference, provided there is no congenital anomaly or severe growth delay.

A major finding of the study was that the dating formulae derived from singleton gestations can be used for dating multiple pregnancies. A simple average of the gestational age prediction of the twins was found to be an accurate predictor of age. In the case of triplets, one day can be added to the average of the longest and shortest gestational age prediction among the triplets. This approach is based upon the analysis of systematic error of formulae derived from singletons when applied to twins and triplets. Our observations imply that fetuses of twin gestations grow at a similar rate to singleton gestations during the midtrimester. However, data on

triplets between 20 and 22 weeks are suggestive of a deceleration of growth, an issue that requires further investigation. We observed that the difference in the gestational age prediction among members of a multiple gestation averaged 2.2 days for twins (maximum = 7.2 days) and 4.2 days for triplets (maximum = 10.8 days). Therefore, when difference among members of multiple gestation is two weeks or more, a search for fetal growth disorders, congenital anomalies, or measurement error is justified.

PERINATOLOGY RESEARCH BRANCH

Chief
Roberto Romero, M.D.

Senior Fellows
Neil Athayde, M.D.
Fabio Ghezzi, M.D.
Ricardo Gomez, M.D.

Guest Researchers
Fernando Arias, Ph.D.
Frank A. Chervenak, M.D.
Frank Hertelendy, Ph.D.
Peter Nathanielsz, Ph.D.
Tushar Niyogi, M.D.
Falah Shamsa, Ph.D.
Marjorie Treadwell, M.D.

Post-Doctoral Fellows
Cristina David, M.D.
Percy Pacora, M.D.

Senior Technical Specialist
Samuel Edwin

ANNUAL BIBLIOGRAPHY

Elder MG, Romero R, Lamont RF. Preterm Labor. London: Churchill Livingstone, 1997.

Fidel PL Jr, Romero R, Cutright J, Wolf N, Gomez R, Araneda H, Ramirez M, Yoon BH. Treatment with the interleukin-I receptor antagonist and soluble tumor necrosis factor receptor Fc fusion protein does not prevent endotoxin-induced preterm parturition in mice. J Soc Gynecol Investig 1997;4:22-6.

Gomez R, Romero R, Edwin SS, David C. Pathogenesis of preterm labor and preterm premature rupture of membranes associated with intraamniotic infection. Infect Dis Clin North Am 1997;11:135-76.

Quintero RA, Romero R, Reich H, Goncalves L, Johnson MP, Carreno C, Evans MI. In utero percutaneous umbilical cord ligation in the management of complicated monochorionic multiple gestations. Ultrasound Obstet Gynecol 1996;8:16-22.

Quintero RA, Reich H, Romero R, Johnson MP, Goncalves L, Evans MI. In utero endoscopic devascularization of a large chorioangioma. Ultrasound Obstet Gynecol 1996;8:48-52.

Svinarich DM, Gomez R, Romero R. Detection of human defensins in the placenta. Am J Reprod Immunol 1997;38:252-55.

Svinarich DM, Wolf NA, Gomez R, Gonik B, Romero R. Detection of human defensin 5 in reproductive tissues. Am J Obstet Gynecol 1997;176:470-5.

Yoon BH, Jun JK, Romero R, Park KH, Gomez R, Choi JH, Kim IO. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. Am J Obstet Gynecol 1997;177:19-26.

Yoon BH, Kim CJ, Romero R, Jun JK, Park KH, Choi ST, Chi JG. Experimentally induced intrauterine infection causes fetal brain white matter lesions in rabbits. Am J Obstet Gynecol 1997;177:797-802.

Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, Kim BI. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol 1997;177:825-30.

Yoon BH, Romero R, Kim CJ, Koo JN, Choe G, Syn HC, Chi JG. High expression of tumor necrosis factor-alpha and interleukin-6 in periventricular leukomalacia. Am J Obstet Gynecol 1997;177:406-11.

Yoon BH, Romero R, Yang SH, Jun JK, Kim IO, Choi JH, Syn HC. Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. Am J Obstet Gynecol 1996;174:1433-40.

INDEX OF TENURED AND TENURE-TRACK INVESTIGATORS

ABBREVIATIONS

Å	Ångstrom
AA	arachidonic acid
9-AA	9-aminoacridine
ACh	acetyl choline
ACTH	adrenocorticotropic hormone
ADNF	activity-dependent neurotrophic factor
ADP	adenosine monophosphate
ADSR	activity-dependent synapse reduction
AFT1	activator of ferrous transport 1
AHP	afterhyperpolarization
Ala	alanine
ALAS	aminolevulinic acid synthase
ALS	acid-labile subunit
AM	adrenomedullin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate
ANP	atrial natriuretic peptic
Ang II	angiotensin II
4-AP	4-aminopyridine
AP	alkaline phosphatase
APs	action potentials
ARF	ADP ribosylation factor
Arg	arginine
Asn	asparagine
Asp	aspartic acid
α1AT	α1-antitrypsin
AT	angiotensin
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BFA	Brefeldin A
BHK	baby hamster kidney cells
bFGF	basic fibroblast growth factor
bp	base pair
BrdU	bromodeoxyribouridine
cAco	cytosolic aconitase
CAH	congenital adrenal hyperplasia
CaM	calmodulin
cAMP	cyclic adenosinemonophosphate

CBMB	Cell Biology and Metabolism Branch
CBP	CREB binding protein
CCD	charge-coupled device
ccg	cell cycle gene
CDC	Centers for Disease Control
CDGS	carbohydrate-deficient glycoprotein syndrome
CDI	color Doppler imaging
CDK	cyclin-dependent kinase
CDP	cytidine diphosphate
C/EBP	CCAAT/enhancer binding protein
GC	cortical granule
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CHO cell	Chinese hamster ovary cell
CICR	calcium-induced calcium release
CID	collision-induced dissociation
CK	casein kinase
cM	centiMorgan
C_m	cell membrane capacitance
CN	Crigler-Najjar
CNS	central nervous system
βCOP	β-subunit of the coatomer complex
CRADA	Cooperative Research and Development Agreement
CREB	cyclic AMP response element binding
CRF	corticotropin releasing factor
CRFR	corticotropin releasing factor receptor
CSF	cerebrospinal fluid
CTHBP	cytosolic TH binding protein
CTR1	gene encoding a copper transporter
CVS	chorionic villous sampling
CYP17	cytochrome P450-17α
Cys	cystine
CZE	capillary zone electrophoresis
DAG	diacylglycerol
DAB	dimethyl ammoniumbromide
DBD	DNA-binding domain
DCBDC	Division of Cancer Biology, Diagnosis, and Centers
DDB	DNA damage-specific, DNA binding
DEANO	1,1-diethyl-2-hydroxy-2-nitrosohydrazine
DEB	Developmental Endocrinology Branch
DHEA	dehydroepiandrosterone
DHFR	dihydrofolate reductase gene
DIC	differential interference camera
DLCO	diffusing capacity of carbon monoxide
ds	double-stranded
DTP	diphtheria/tetanus/pertussis
E	embryonic day
EBV	Epstein-Barr virus
EC	entorhinal cortex
EC cells	embryonal carcinoma cells
ECM	extracellular matrix
EDS	Ehlers-Danlos syndrome
EE	ethynyl estradiol
eEF	eukaryotic elongation factor
EGF	epidermal growth factor
egt	ecdysone UDP-glucosyltransferase
EGTA	ethylene glycol tetraacetic acid
eIF	eukayotic initiation factor
EPSP	excitatory post-synaptic potential
ER	endoplasmic reticulum
ERE	estrogen response element
ERRB	Endocrinology and Reproduction Research Branch
ES	embryonal stemcell
EST	estrogen sulfotransferase
est	expressed sequence tags
ET	endothelin
FAK	focal adhesion kinase
FDA	Food and Drug Administration
FEV_1	forced expiratory volume in 1 second
FGF	fibroblast growth factor
FISH	fluorescence *in situ* hybridization
FLIP	fluorescence loss in photobleaching
FMPP	familial male precocious puberty
F_2Pmp	difluorophosphonomethyl phenylalanine
fPS	field potential
FRE	gene encoding ferric reductase
FRGY	frog Y-box protein
FSH	follicle-stimulating hormone
Ft H	ferritin heavy chain
GABA	gamma-aminobutyric acid
galtf	galactosyl transferase
GCD	general control derepressed
GCN	general control non-derepressible
GDNF	glial cell line-derived neurotrophic factor
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone releasing hormone
Gln	glutamine
Glu	glutamic acid
GluR	glutamate receptor
GMP	guanosine monophosphate
GnRH	gonadotropin releasing hormone
G6Pase	glucose-6-phosphatase
GPCR	G protein-coupled receptor
GPI	glycosyl phosphatidylinositol
GR	glucocorticoid receptor
GSD	glycogen storage disease
GSK	glycogen synthase kinase
GST	glutathione-S-transferase
GT	glucose transporter
GTP	guanosine triphosphate
HA	hemagglutinin
HAT	histone acetyltransferase
hCG	human chorionic gonadotropin
HD	histone deacetylase
HDB	Heritable Diseases Branch
HDF	human diploid fibroblast
5-HIAA	5-hydroxyindoleacetic acid
HIb	*Haemophilus influenz*ae type b
His	histidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLUG	human liver bilirubin UDP-glucuronosyltransferase
HPA	hypothalamic pituitary adrenal axis
HPLC	high pressure liquid chromatography
HPS	Hermansky-Pudlak syndrome
HPV	human papilloma virus
HSD	hydroxysteroid dehydrogenase
HSP	heat shock protein
HST	hydroxysteroid sulfotransferase
5HT	serotonin (5-hydroxytryptamine)
HUG-Br1	human bilirubin UDP-glucuronosyltransferase
IAM	iodoacetamide
ICSBP	interferon consensus sequence binding protein
icv	intracerebroventricular
IDE	insulin-degrading enzyme
IDF	Israeli Defense Forces
IEG	immediate early gene
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IGFBP	IGF binding protein
IL	interleukin
Ile	isoleucine
IN	integrase
IND	investigational new drug
INH	isonicotinic acid hydrazide (isoniazid)

Inr	initiator element
Ins	inositol
IPCR	inositol triphosphate receptor
IPSS	inferior petrosal sinus sampling
IRE	iron responsive element
IRF	interferon regulatory factor
IRP	iron regulatory protein
IRR	insulin receptor-related receptor
IVF	*in vitro* fertilization
ISRE	interferon-stimulated response element
ITAM	immunoreceptor tyrosine-based activation motif
kb	kilobase
kDa	kilo Dalton
LCE	Laboratory of Comparative Ethology
LCH	Leydig cell hypoplasia
LCM	Laser Capture Microdissection
LCMB	Laboratory of Cellular and Molecular Biophysics
LCMN	Laboratory of Cellular and Molecular Neurophysiology
LDMI	Laboratory of Developmental and Molecular Immunology
LDN	Laboratory of Developmental Neurobiology
LEGR	Laboratory of Eukaryotic Gene Regulation
Leu	leucine
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LHRH	luteinizing hormone releasing hormone
LIMB	Laboratory of Integrative and Medical Biophysics
LME	Laboratory of Molecular Embryology
LMG	Laboratory of Molecular Genetics
LMGD	Laboratory of Mammalian Genes and Development
LMGR	Laboratory of Molecular Growth Regulation
LMP	last mestrual period
lod	log of the odds
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LT	leukotriene
LTD	long-term depression
LTP	long terminal repeat
LTP	long-term potentiation
Lys	lysine
mAco	mitochondrial aconitase
MARCKS	myristoylated alanine-rich PKC substrate
MBP	maltose binding protein
MBT	mid-blastula transition
MIIC	MHC class II compartment
MCA	middle cerebral artery
MDR	multi-drug resistance
Met	methionine
MeV	megaelectronvolt
MHC	major histocompatibility complex
MLTC	mouse Leydig tumor cell
MMP	matrix metalloproteinase
MMTV	murine mammary tumor virus
M_r	molecular weight
MRI	magnetic resonance imaging
MS	multiple sclerosis
MT	metallothionein
MTOC	microtubule organizing center
MuLV	murine leukemia virus
mV	millivolt
NASA	National Aeronautics and Space Administration
NC	nucleocapsid
NCAM	neural cell adhesion molecule
NCHGR	National Center for Human Genome Research
NCI	National Cancer Institute
NE	norepinephrine
NEM	N-ethylmaleimide
NER	nucleotide excision repair
NFI	nuclear factor I
Ng	neurogranin
NGF	nerve growth factor
NIA	National Institute of Aging
NIAID	National Institute of Allergy and Infectious Diseases
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIDDM	noninsulin-dependent diabetes mellitus
NIDR	National Institute of Dental Research
NIHAC	NIH Animal Center
NIMH	National Institute of Mental Health
NINDS	National Institute of Neurological Disorders and Stroke
NHLBI	National Heart, Lung and Blood Institute
NK cells	natural killer cells
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NO	nitric oxide
NOG	neurite outgrowth
nS	nanoSiemens
NSF	NEM-sensitive fusion protein
NSI	non-syncytium-inducing
nt	nucleotide
OBR	origins of bidirectional replication (OBR)
OCA	oculocutaneous albinism
OI	osteogenesis imperfecta
OPN	osteopontin
ORF	open reading frame
P	postnatal day
P1	promoter 1
pA	polyadenylation
PA	phosphatidic acid
PACAP	pituitary adenylyl cyclase activating peptide
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PBMC	peripheral blood mononuclear cells
PC	phosphatidylcholine
PCE	pro-opiomelanocortin converting enzyme
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethyleneglycol
PEPA	4-[2-(phenylsulfonylamino) ethylthio]-2,6-difluoro-phenoxyacetamide
Pet	phosphatidylethanol
PET	positron emission tomography
pF	picofarad
PG	phosphatidylglycerol
PHA	phytohemagglutinin
Phe	phenylalanine
PhTX	Philanthotoxin 343
pI	isolectric point
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-phosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKR	RNA-regulated protein kinase
PLA	phospholipase
PMA	phorbol methyl acetate
PN	protease nexin
POMC	pro-opiomelanocortin
PPT	polypurine tract
PRB	Perinatology Research Branch
PRG	primary response gene
PRLR	prolactin receptor
Pro	proline
PS	phosphatidylserine
PSG	pregnancy-specific glycoprotein
PtdIns	phosphatidyl inositol
PTH	parathyroid hormone
PTK	protein tyrosine kinase

PVL	periventricular leukomalacia
PVM	parasitophorous vacuole membrane
PVN	paraventricular nucleus
RA	retinoic acid
RACE	rapid amplification of cDNA ends
Rag	recombination activation gene
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RAR	retinoic acid receptor
RARE	retinoic acid response element
Rb	retinoblastoma gene
RBP	RNA binding protein
RDS	respiratory distress syndrome
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
RNP	rinonucleoprotein
RRM	RNA recognition motif
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
RWV	rotating wall vessel
RZ	ribozyme
scRNA	small cytoplasmic RNA
SD	standard deviation
SDS	sodium dodecylsulfate
SELEX	systematic evolution of ligands by exponential enrichment
s.e.m.	standard error of the mean
Ser	serine
SERCA	sarcoplasmic endoplasmic reticulum calcium
SES	socio-economic status
SI	syncytium-inducing
SNAP	synaptosomal-associated protein
SNARE	synaptosomal-associated protein receptor
SRP	signal recognition particle
ss	single-stranded
SSCP	single strand conformation polymorphism
st.	*stratum*
ST	stromelysin
STS	sequence-tagged sites
SUMO	small ubiquitin-related modifier
SV40	simian virus 40
TAF	tightly associated factor
TAM	tyrosine based activation motif
TAP	trimethyl ammoniumpropane
TBP	TATA-binding protein
TCR	T cell antigen receptor
t_d	delay time
TEA	triethanolamine
TF	transcription factor
TGN	*trans*-Golgi Network
TH	thyroid hormone
Thr	threonine
TIK	tyrosine immunoreactive kinase
TM	transmembrane
TNF	tumor necrosis factor
TPA	phorbol-12-myristate-13-acetate
TnI	troponin I
TR	thyroid hormone receptor
TRAP	thrombin receptor activating peptide
TRAP	twin reversal arterial perfusion
TRE	thyroid hormone response element
TRH	thyrotropin-releasing hormone
Trp	tryptophan
ts	temperature-sensitive
TSA	Trichostatin A
TSS	transcriptional start site
TVS	tubulo-vesicular structure
Tyr	tyrosine
UDP	uridine diphosphate
UG	uteroglobin
UGT	UDP-glucuronosyltransferase
USUHS	Uniformed Services University of the Health Sciences
UTR	untranslated region
Val	valine
VAMP	vesicle-associated membrane protein
VBC	heterotrimer of VHL, Elongin B, and Elongin C
VCP	valosin-containing protein
VDAC	voltage-dependent anionic channel
VDR	vitamin D receptor
VEGF	vesicular endothelial growth factor
VHL	von Hippel-Lindau
VIBP	VSV-induced binding protein
VIP	vasoactive intestinal peptide
V_m	membrane potential
VP	vasopressin
VSCC	voltage-sensitive Ca^{2+} channel
VSV	vesicular stomatitis virus
VV	*vaccinia* virus
WRAIR	Walter Reed Army Institute for Research
WT	wortmannin
YAC	yeast artificial chromosome
YAP3	yeast aspartic protease 3
XHSF	*Xenopus* heat shock transcription factor
XP	*Xeroderma pigmentosum*
XSCID	X-linked severe combined immunodeficiency
ZAP	zeta-associated protein

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health